Tocopherols
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MONOGRAPH
ON THE
TOCOPHEROLS

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TOCOPHEROLS

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TOCOPHEROLS

Summary

A factor required in animal nutrition as a dietary constituent necessary for normal reproduction has been termed vitamin E. This factor is generally considered an essential nutrient for man, but this has not yet been definitely proven. Although alpha-tocopherol is often used synonomously with vitamin E, vitamin E activity is actually a quality possessed by other tocopherols as well as alpha-tocopherol, for example:

1 mg dl-alpha-tocopherol acetate = 1 internation unit of vitamin E activity

1 mg dl-alpha-tocopherol = 1.1 I.U.

1 mg d-alpha-tocopherol acetate = 1.36 I.U.

1 mg d-alpha-tocopherol = 1.49 I.U.

The tocopherols, but not alpha-tocopherol acetate, are widely distributed in organic materials and are therefore a regular part of the human diet.

Alpha-tocopherol is readily absorbed from the gastrointestinal tract (1283, 1902, 2136, 1532, 255). L-alpha-tocopherol is absorbed and excreted more rapidly than d-alpha-tocopherol (2550), and there is a synergism between the two forms in absorption (2546). The other tocopherols and alpha-tocopherol acetate are absorbed less readily than alpha-tocopherol (1902, 909). According to Gallo-Torres (837) bile is essential for the intestinal absorption of alpha-tocopherol acetate and pancreatic juice is necessary for its hydrolysis. The absorption of alpha-tocopherol reportedly occurs mainly via the lymphatic pathway (1533, 1218, 255), with small amounts being transported from the intestine via the portal vein (1533). The distribution of alpha-tocopherol throughout the body is extensive (1375, 1655, 888) and intracellular vitamin E is found predominantly in the mitochondrial and microsomal fractions (1375).

Several groups of workers have studied the metabolism of the tocopherols, but so far their conclusions differ. On the basis of studies using rabbits, Kaludin (1244) suggested that beta-, gamma-, and delta-tocopherol are converted to alpha-tocopherol in the body. In a study employing hens, Dju et al. (632) were unable to confirm this suggestion. In studies on rabbits and human subjects, Simon et al. (2241, 2242) were able to isolate two metabolites of dl-alpha-tocopherol, 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone and its gamma-lactone. Schmandke (2130, 2131) reported that in man alpha-tocopherol is converted, to a small extent only, to tocopheronolactone. Alaupovic et al. (29) found that alpha-tocopherol is converted in rats and pigs into 3 metabolites, which they designated compounds F, Oi, and Os.

The concentrations of ATP, ADP, and CoA in the livers of male albino rats were found by Gray et al. (931) to be significantly elevated by the addition of 100 mg of alpha-tocopherol acetate daily to the diet of the rats for 20 days.

Smolinska (2268) reported that oral administration of vitamin E affects the concentrations of Cu and Co in the liver, muscles, and bones of rats, the direction of effect depending upon the dose.

Gray and Loh (930) noted significant increases in phospholipids, total cholesterol, and alpha-2 globins and significant decreases in amino acids in the plasma of healthy medical students following daily ingestion of 100 mg of alpha-tocopherol acetate for 10 days. Liver phospholipids and total and ester cholesterol were found by Gray (926) to be increased in rats given 100 mg alpha-tocopherol acetate per day for 8 days. However, Marx et al. (1592) reported that hypervitaminosis E had no effect on the blood or tissue cholesterol levels of rats, although it did product localized sclerosis and deposition of cholesterol in the aorta.

The coagulation time and prothrombin time of blood taken from 23 human subjects following the oral administration of 100 mg of Ephinal were determined by Prosperi (1977) to be shortened. Feruglio and Petrin (775) observed a trend toward hypercoagulation in the blood taken from men who had received 100 mg alpha-tocopherol daily for 10 days. Tul'chinskays and Mishnaevskiy (2438) reported that the feeding by vitamin E to chicks increased the output of normal antigen agglutinins and the titer of blood complement, and stimulated the phagocytic activity of the leukocytes. March et al. (1559) found that the addition of dl-alpha-tocopherol (120 IU/kg) to the diet of chicks resulted in an increase of the number of circulating reticulocytes.

Ichihara (1152) reported that the administration of vitamin E to pregnant mice activates the secretory function of GH, LTH and LH in the anterior pituitary.

Studies by Baroni and Casa (125) disclosed that the administration of alpha-tocopherol can increase the basal metabolic rate in man.

Szarka (2347) induced estrus in infantile rats by administration of substances containing high levels of vitamin E.

Miles et al. (1685) and Dattatreya Rao et al. (537) reported that alpha-tocopherol exhibits a vitamin A-sparing effect. Kryukova and Pechenina (1385) and Maddock et al. (1535) reported that vitamin E potentiates the adverse effects of hypervitaminosis A in rats. McCuaig and Matzok (1636) stated that dl-alpha-tocopherol acetate prevented the adverse effects of hypervitaminosis A.

Gazet du Chatelier (848) reported that dl-alpha-tocopherol administered at high dosage (50 mg/kg) sensitized the guinea pig to histamine. Haber and Wissler (984) found that increasing the dietary intake of alpha-tocopherol 100-fold or more above the normal intake markedly reduced the incidence of tumor in mice

treated with methylcholanthrene. Gallagher (836) found that the prior or simultaneous administration of alpha-tocopherol acetate (55 IU) afforded protection to rats given lethal doses of CCl_4.

Tests of the carcinogenicity of vitamin E have produced contrasting results. For a period of 7 months, Telford (2381) maintained 200 mice, which had been given 1 mg of (1,2,4,6)dibenzanthrene in 0.25 ml olive oil to induce lung tumors, on normal or vitamin E-deficient diets with or without a supplement of alpha-tocopherol (2 mg every other day). Autopsies at the end of 7 months revealed the incidence of lung tumor to be greatest in the hypervitaminosis groups. Tests by Demole (573) on the mouse and rat indicate that dl-alpha-tocopherol has no carcinogenic properties. In a study by Shamberger and Rudolph (2192), the incidence of tumor formation in mice treated with 7,12-dimethylbenzanthracene and croton oil was reduced by the administration of alpha-tocopherol. Kochur et al. (1337) reported that the administration of alpha-tocopherol or its acetate to rats with various transplanted tumors retarded tumor growth and prolonged survival.

The delayed or tuberculin type of sensitization has been produced by Lipton (1496) in guinea pigs; alpha-tocopherol acetate failed to produce sensitization.

A great number of studies on the feeding of vitamin E have been performed. However, the majority of these studies are concerned with vitamin E deficiency and therefore are not relevant to this work. Only a small number of studies on the effects of excess vitamin E are available, none of which are long-term. Only a very limited amount of information on the acute toxicity of tocopherol is available and is presented in the table on page 28. A summary of the short-term studies follows.

Ichihara (1152) reported that the daily treatment of mice with 10 mg dl-alpha-tocopherol acetate caused enlargement of the testicular interstitial cells and marked development of the smooth endoplasmic reticulum, indicating that vitamin E administration activates the function of steroid hormone production in the testicular interstitial cells.

Hill and Hamed (1083) administered, parentally, a total of 2000 mg of dl-alpha-tocopherol acetate per animal to growing chicks over a period of 10 weeks. A distinct growth inhibition was observed in the chicks and the development of the primary and secondary sex characteristics was significantly retarded (1083). As a result of feeding growing chicks a diet containing 1% dl-alpha-tocopherol acetate for 32 days, McCuaig and Metzok (1636) observed the livers of the treated chicks to be larger and darker red than those of the controls. The administration of 5000 I.U. of alpha-tocopherol acetate to day-old chicks for 21 days inhibited the growth rate, while 500 I.U./kg exhibited no effect (1761).

Doses of 100 or 500 mg synthetic or natural alpha-tocopherol per day had no effect on female rats or their offspring during gestation or lactation (1761). Telford et al. (2384) determined that tocopherols have no significant effect on the resorption rate

in rats. Lehr et al. (1463) reported that excessive amounts of alpha-tocopherol (10 mg/day plus 100 mg/100 g body weight 3 times weekly for 4 weeks) appeared responsible for an increased incidence of advanced lesions in the aorta, coronary arteries, and stomach and moderate hypertension in rats. In groups of rats given d- or dl-alpha-tocopherol orally in doses of 200, 700, and 2000 mg/kg 5 days per week for 3 months, the thyroids showed slight hyperplasia at the highest dose; no other adverse effects were observed (1761). On the basis of a 98-day feeding study, it has been concluded that the daily intake per rat of 460 mg of alpha-tocopherol is without effect (1761). The administration to rats of 1 g of deodorized refined cottonseed oil (Wesson oil) or Alpha Tocopherol Concentrate (255 mg total tocopherols/g) per day 5 days per week for 105 days produced no adverse effects (1761). Tonkelnar et al. (2419) reported that the addition of 0.002 or 0.004% alpha-tocopherol to a 15% lard diet of rats for 13 weeks caused no untoward effects attributable to the ingestion of alpha-tocopherol. Katsui and Ima (1265) found that the growth and appearance of rats receiving 0.50 mg of dl-alpha-tocopherol/day for 40 days was completely normal. Demole (572) reported that the administration of dl-alpha-tocopherol on 10 consecutive days was tolerated by the male rat in the dose of a total of 1 g/kg orally, while the normal female rat tolerated 0.5 g/kg without symptoms. Dl-alpha-tocopherol administered for a period of two months orally to a group of 10 male and 10 female rats in a total dose of 4 g/kg had no effect on the reproduction of the rats (572). The growth and reproduction of rats given 20 mg dl-alpha-tocopherol daily for 49 days was observed by Demole (573) to be unaffected.

Escudera and Herraiz (723) reported that male rats given 30 micrograms alpha-tocopherol acetate per day for 25 weeks exhibited little symptoms of testicular atrophy while those receiving smaller doses exhibited extensive testicular atrophy and 66% of those receiving 600 micrograms/day showed slight histological lesions of the testicles. Gray et al. (932) reported that administration of 100 mg alpha-tocopherol acetate/day to rats for 4-6 weeks produced an increase in liver weight and a significantly higher concentration of DNA in the liver. The feeding of weanling male rats with a diet supplemented with 500 or 5000 IU alpha-tocopherol acetate/kg resulted in a significant decrease in liver weight (1761). Five weanling female rats given 200 mg alpha-tocopherol acetate per day for 14 weeks exhibited significant growth depression and 3 of the rats died within 2 weeks (1761). No such effects were observed for rats given 100 mg alpha-tocopherol acetate or less (1761). Mating of these rats revealed no adverse effects on reproduction (1761). In an experiment in which male rats were given, for 105 days, 1 g/day (5 days per week) of deodorized, refined cottonseed oil (Wesson Oil) or Alpha Tocopherol Acetate Concentrate (200 mg tocopherol acetate/g), no adverse effects were observed (1761). Dl-alpha-tocopherol acetate administered for a period of two months orally to rats in a total dose of 4 g/kg was well tolerated and had no adverse effect on reproduction (573). The administration of dl-alpha-tocopherol acetate on 10 consecutive days was well tolerated by the male rat in the dose of a total of 1 g/kg orally, while the normal female rat tolerated 0.5 g/kg without any symptoms (573).

Czyba (508) reported that subcutaneous injection of 75 mg of dl-alpha-tocopherol acetate per day to hamsters for 8-30 days caused a decrease in the weight of the testicles and impairment of spermatogenis. These effects seem to be transitional since spermatogenesis was restored after 3 weeks (506).

Costa et al. (487) observed intense hyperemia of the thyroid in guinea pigs which consumed 13 mg of alpha-tocopherol per day for 10 days.

Prosperi and Borselli (1981) reported that treatment of guinea pigs with large intramuscular doses of vitamin E, injected either in single or multiple doses, caused degenerative lesions involving the liver and, to a lesser extent, the myocardium and encephalic substance. A study of the endocrine system of guinea pigs during E hypervitaminosis revealed serious damages of adrenal gland and incretory stimulation in the pancreas, and especially in the hypophysis, testicles and ovaries (1981).

Following oral administration of 5.5-12.5 mg tocopherol acetate/kg of body weight/day to 4 male and 6 female rabbits for 1-3 months, Huter (1141) observed highly active thyroids, with normal iodine protein contents, in the females and abnormal growth of the thyroids with absence of or deficiency in iodine and enhanced epithelial growth in the males.

In the treatment of lupus erythematosus, Welsh (2564) administered various daily dosages of vitamin E in conjunction with pantothenic acid derivatives to 36 patients for periods ranging from 2 to 36 months. Eleven of the patients consumed over 200 g each in the course of the treatment and one patient was given 424.2 g of vitamin E within 20 months (2564). In no case were any complications or side effects observed (2564). Hillman (1085) reported that the ingestion of 296 g of alpha-tocopherol by a 41-year-old man over a period of 93 days produced an elevated plasma tocopherol level and a transitory creatinuria. Winkler (2595) reported that oral administration of 30 or 300 mg of alpha-tocopherol per day resulted in disturbances of the rhythm of the estrus cycle as well as prolan, estrone, and estriol excretion in girls.

Greenblatt (946) gave 6 male subjects 40 g d-alpha-tocopherol-acetate per day for periods as long as 1 month without producing any adverse effects. For intervals up to 3 years, Welsh (2570) administered 1-2 g of alpha-tocopherol acetate daily, in conjunction with various doses of pantothenic acid derivatives to 67 patients who had lupus erythematosis. Transient nausea and gastric distress were the only effects resultant from the treatment (2570). Costa (487) reported that the daily introduction of 400-500 mg alpha-tocopherol acetate induces, in the normal individual, metaholic conditions like those which are found in cases of hyperthyrosis - decrease of the basal metabolism in 58% of patients, increase of thyroid iodine uptake, and increase of serous organic iodine.

ALPHA-TOCOPHEROL

Chemical Information

I. Nomenclature

A. Common Names

1. Alpha-tocopherol
2. Vitamin E
3. Antisterility vitamin
4. 5,7,8-trimethyltocol

B. Chemical Names

1. 2,5,7,8,-Tetramethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol

C. Trade Names

1. Eprolin-S
2. Epsilan
3. Ephynal
4. Syntopherol
5. E-Vimin
6. Evipherol
7. Etavit
8. Phytogermine
9. Profecundin
10. Tokopharm
11. Viteolin
12. Esorb
13. Vascuals
14. Covitol
15. Evion

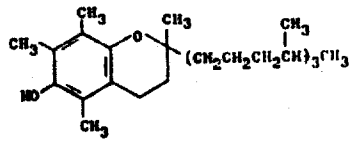
D. Chemical Abstracts Registry Number

1. Alpha Tocopherol - 000 059 029
2. D,L-Alpha Tocopherol - 000 364 501
3. Tocopherols - 001 406 662
4. Tocopherols concentrate, mixed - 977 009 178

II. Empirical Formula

$C_{29}H_{50}O_2$

III. Structural Formula



IV. Molecular Weight

430.69

V. Specifications

Food Chemicals Codex

Assay	Not less than 97.0% $C_{29}H_{50}O_2$
Refractive Index	Between 1.503 and 1.507 at 20°
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm
Heavy metals (as Pb)	Not more than 40 ppm
Lead	Not more than 10 ppm

VI. Description

A. General characteristics

The tocopherols are pale yellow, viscous oils at room temperature.

B. Physical properties

Alpha-tocopherol is freely soluble in oils, fats, acetone, alcohol, chloroform, ether and other fat solvents and is practically insoluble in water. It exhibits the following characteristics: mp 2.5-3.5 degrees; $bp_{0.1}$ 200-220 degrees; d25/4 0.950; n25/D 1.5045; absorption maximum 294 nm (E 1%/1cm 71).

C. Stability

Alpha-tocopherol is stable to heat and alkalies in the absence of oxygen; in the presence of oxygen it is readily degraded by heat, particularly at temperatures employed in refining and processing vegetable fats. Alpha-tocopherol is not affected by acids up to 100 degrees. It is oxidized slowly by atmospheric oxygen and rapidly by ferric and silver salts. Exposure to light causes alpha-tocopherol to gradually darken. It is not precipitated by digitonin.

VII. Analytical Methods

The estimation of the vitamin E content of foods and food supplements is intricate. The chief problem besetting the analyst is the separation of the tocopherols from their associated contaminants. The isolation of the tocopherols from natural products has been partially accomplished by various combinations of such procedures as extraction, molecular distillation, saponification, hydrogenation, adsorption chromatography, and sulfuric acid wash (1025).

Most determinations of the total tocopherol content of purified extracts of foods are based upon the Emmerie and Engel reaction, which involves the reaction of the tocopherols in the extract with ferric chloride to yield ferrous chloride. The ferrous chloride reacts with

alpha, alpha-dipyridyl to yield a red complex which is measured colorimetrically. However, these reagents will react with any reducing material still remaining in the food extract. Thus, the validity of total tocopherol values is dependent upon the success of the purification procedures employed prior to application of the nonspecific Emmerie and Engel reaction (1025).

The official method for the determination of naturally occurring alpha-tocopherol in foods and feeds involves extraction with hot ethanol, saponification, isolation of alpha-tocopherol by TLC and colorimetry (1025).

Use of the nitroso assay for the estimation of alpha-tocopherol was formerly widely utilized. It depends on the ability of some of the non-alpha-tocopherols to form colored derivatives. Alpha-tocopherol does not form a colored derivative and thus any unremoved reducing materials would be termed alpha-tocopherol. When applied to natural products and foods, the nitroso procedure often considerably overestimates the amount of alpha-tocopherol present (1025).

The separation of alpha-tocopherol from other tocopherols and other reducing substances can be achieved by the use of paper chromatography employing paper impregnated with either Vaseline or paraffin. By using zinc-amine impregnated paper, further separation of the non-alpha-tocopherols can be accomplished. In spite of the small amounts of tocopherols used and the necessity to calibrate for losses during chromatography, paper has been extensively used for the determination of alpha-tocopherol in foods and other natural products (1025).

A multiple-solvent column chromatographic procedure has been found to give good results. The lipid extract of a food is eluted through an activated dibasic magnesium phosphate column with a series of solvents containing progressively increased amounts of diethyl ether in petroleum ether. Carotenoids are eluted with 0.5% diethyl ether, and 2.0% diethyl ether specifically elutes the alpha-tocopherol and trimethyltocotrienol. Thus, the presence of trimethyltocotrienol will lead to an overestimation of alpha-tocopherol content, but this is not critical because trimethyltocotrienol is not widely distributed in significant amounts. The primary difficulty with this procedure is obtaining a satisfactory source of dibasic magnesium phosphate. Only by carefully checking the elution properties of each lot of activated adsorbant with a mixture of alpha- and gamma-tocopherols can one be assured of reliable results. This procedure is more rapid than two-dimensional paper chromatography and has been used for the routine determination of alpha-tocopherol in foods (1025).

The technique of thin-layer chromatography (TLC) has been successfully adapted to the separation and analysis of the tocopherols. An extract containing the tocopherols is placed on a plate coated with a thin-layer of silica gel or alumina, developed with organic solvents and the spot of alpha-tocopherol is extracted and determined colorimetrically. TLC on silica gel has been used for the determination of alpha-tocopherol in plant tissues and in vegetable oils. A procedure for the determination of alpha-tocopherol in foods has been reported using alumina CHROMAGRAM sheets suitable for routine analyses.

The substitution of bathophenanthroline for alpha,alpha-dipyridyl in the Emmerie and Engel reaction increased the sensitivity 2.5-fold and is especially useful for the small amounts of alpha-tocopherol isolated from TLC plates and sheets (1025).

Gas-liquid chromatographic systems for the separation of the tocopherols have been described and, with modifications, applied to the identification of alpha-tocopherol in mixtures, in animal tissue extracts, and in some foods. Sterols tend to interfere in the determination and must be carefully removed. Because of its speed, sensitivity, and high resolving power, this technique may ultimately find extensive application for the determination of alpha-tocopherol in foods and food supplements (1025).

VIII. Occurrence

A. Plants

The various tocopherols are found almost exclusively in plants, in which alpha-tocopherol seems to prevail. They are present in highest concentration (0.1-0.3%) in wheat germ, corn, cottonseed, sunflower seed, rapeseed, and soybean oils, in which they occur in unesterified form. Lettuce and alfalfa also contain considerable amounts of the tocopherols.

B. Animals

The various tocopherols are found only to a minimal degree in animal tissues. Meat, liver, milk, and eggs are the richest animal sources.

C. Synthetics

The tocopherol carbon skeleton has been built up by six different types of approaches, schematically deplicted in Fig. 1 (1025).

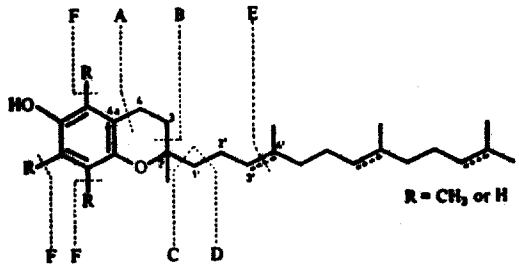


FIG. 1. Schemes of tocopherol syntheses. Scheme A. Formation of the C_4—C_{4a}-bond by condensation of an appropriate hydroquinone or a derivative thereof with phytol or a derivative thereof. Scheme B. Formation of the C_2—C_3-bond by condensation of an appropriate phenethyl derivative with the C_{18}-ketone V. Scheme C. Formation of the C_2—C_1-bond by condensation of an appropriate benzylacetone derivative with a C_{16}-component. Scheme D. Formation of the C_1—C_2-bond by condensation of an appropriate chromanol derivative with an isoprenoid C_{15}-component. Scheme E. Formation of the C_3—C_4-bond by condensation of an appropriate chromanol derivative with an isoprenoid C_{13}-component. Scheme F. Introduction of methyl groups at C_5 and (or) C_7 and (or) C_8 into tocols or tocotrienols by chloromethylation, aminomethylation, formylation, or hydroxymethylation procedures.

D. Natural inorganic sources

None

BETA-TOCOPHEROL

Chemical Information

I. Nomenclature

A. Common Names

1. Beta-tocopherol
2. 5,8-Dimethyltocol
3. Cumotocopherol
4. Neotocopherol
5. p-Xylotocopherol

B. Chemical Name

1. 2,5,8-Trimethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol

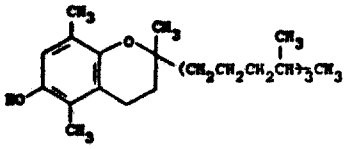
C. Trade Name

None

II. Empirical Formula

$C_{28}H_{48}O_2$

III. Structural Formula



IV. Molecular Weight

416.66

V. Specifications

Not available

VI. Description

A. General Characteristics

See alpha-tocopherol

B. Physical Properties

Beta-tocopherol shows solubilities similar to alpha-tocopherol.

It exhibits the following characteristics: $bp_{0.1}$ 200-210°; absorption maximum 297 nm; E 1%/1 cm 86.4.

C. Stability

Same as alpha-tocopherol

VII. Analytical Methods

See alpha-tocopherol

VIII. Occurrence

A. Plants

Beta-tocopherol accompanies alpha-tocopherol from natural sources (see alpha-tocopherol).

B. Animals

See above

C. Synthetics

See alpha-tocopherol

D. Natural inorganic sources

None

GAMMA-TOCOPHEROL

Chemical Information

I. Nomenclature

A. Common Names

1. Gamma-tocopherol
2. 7,8-Dimethyltocol
3. o-Xylotocopherol

B. Chemical Name

1. 2,7,8-Trimethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol

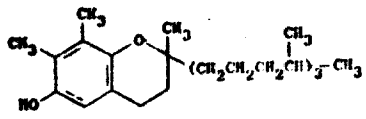
C. Trade Name

None

II. Empirical Formula

$C_{28}H_{48}O_2$

III. Structural Formula



IV. Molecular Weight

416.66

V. Specifications

Not available

VI. Description

A. General Characteristics

See alpha-tocopherol

B. Physical Properties

Gamma-tocopherol shows solubilities similar to alpha-tocopherol. It has the following characteristics: mp -30°; $bp_{0.1}$ 200-210°; absorption maximum 298 nm; E 1%/1 cm 92.8.

C. Stability

Same as alpha-tocopherol

VII. Analytical Methods

See alpha-tocopherol

VIII. Occurrence

A. Plants

Gamma-tocopherol accompanies alpha-tocopherol from natural sources (see alpha-tocopherol).

B. Animals

See above

C. Synthetics

See alpha-tocopherol

D. Natural Inorganic sources

None

DELTA-TOCOPHEROL

Chemical Information

I. Nomenclature

A. Common Names

1. Delta-tocopherol
2. 8-Methyltocol

B. Chemical Name

1. 2,8-dimethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol

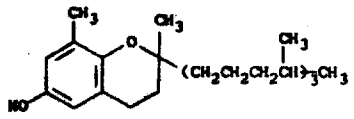
C. Trade Name

None

II. Empirical Formula

$C_{27}H_{46}O_2$

III. Structural Formula



IV. Molecular Weight

402.64

V. Specifications

Not available

VI. Description

A. General Characteristics

See alpha-tocopherol

B. Physical Properties

Delta-tocopherol shows solubilities similar to alpha-tocopherol. It has the following characteristics: absorption maximum 298 nm; E 1%/1 cm 91.2.

C. Stability

Same as alpha-tocopherol

VII. Analytical Methods

See alpha-tocopherol

VIII. Occurrence

A. Plants

Delta-tocopherol comprises 30% of the mixed tocopherols isolated from soybean oil and 5% of that from wheat germ oil. Evidence has been presented indicating the presence of delta-tocopherol in cottonseed oil and peanut oil.

B. Animals

None

C. Synthetics

See alpha-tocopherol

D. Natural inorganic sources

None

EPSILON-TOCOPHEROL

Chemical Information

I. Nomonclature

A. Common Name

1. Epsilon-tocopherol

B. Chemical Name

1. 2,5,8-trimethyl-2-(4',8',12'-trimethyltrideca-3,7,11-trienyl)chroman-6-ol

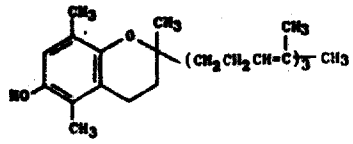
C. Trade Name

None

II. Empirical Formula

$C_{28}H_{42}O_2$

III. Structural Formula



IV. Molecular Weight

410.62

V. Specifications

Not available

VI. Description

A. General characteristics

See alpha-tocopherol

B. Physical Properties

Epsilon-tocopherol shows solubilities similar to alpha-tocopherol. It exhibits the following characteristics: absorption maximum in

ethanol 296 nm (E 1%/1 cm 87).

C. Stability

Same as alpha-tocopherol

VII. Analytical Methods

See alpha-tocopherol

VIII. Occurrence

A. Plant

Isolated from wheat germ oil and bran.

B. Animal

None

C. Synthetics

See alpha-tocopherol

D. Natural inorganic sources

None

ZETA$_1$-TOCOPHEROL

Chemical Information

I. Nomenclature

A. Common Names

1. Zeta$_1$-tocopherol
2. 5,7,8-Trimethyltocotrien-3',7',11'-ol

B. Chemical Names

1. 2,5,7,8-tetramethyl-2(4',8',12'-trimethyltrideca-3,7,11-trienyl)-chroman-6-ol

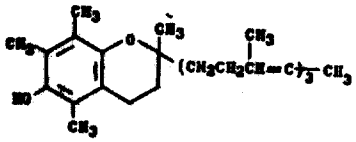
C. Trade Name

None

II. Empirical Formula

$C_{29}H_{44}O_2$

III. Structural Formula



IV. Molecular Weight

424.64

V. Specifications

Not available

VI. Description

A. General Characteristics

See alpha-tocopherol

B. Physical Properties

Zeta$_1$-tocopherol shows solubilities similar to alpha-tocopherol. The absorption maximum in ethanol is 292.5 nm (E 1%/1 cm 91).

C. Stability

Same as alpha-tocopherol

VII. Analytical Methods

See alpha-tocopherol

VIII. Occurrence

A. Plant

Isolated from wheat bran

B. Animal

None

C. Synthetics

See alpha-tocopherol

D. Natural inorganic sources

None

ZETA$_2$-TOCOPHEROL

Chemical Information

I. Nomenclature

A. Common Names

1. Zeta$_2$-tocopherol
2. 5,7-Dimethyltocol

B. Chemical Name

Zeta$_2$-tocopherol

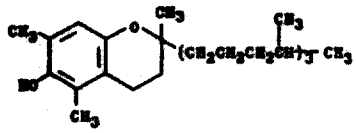
C. Trade Name

None

II. Empirical Formula

$C_{28}H_{48}O_2$

III. Structural Formula



IV. Molecular Weight

416.66

V. Specifications

Not available

VI. Description

A. General Characteristics

See alpha-tocopherol

B. Physical Properties

Zeta$_2$-tocopherol shows solubilities similar to alpha-tocopherol. Crystallizes in prisms at -4 degrees. The absorption maximum in ethanol is 292 nm (E 1%/1 cm 83.0).

C. Stability

Same as alpha-tocopherol

VII. Analytical Methods

See alpha-tocopherol

VIII. Occurrence

A. Plant

Isolated from rice

B. Animal

None

C. Synthetics

See alpha-tocopherol

D. Natural inorganic sources

None

ETA-TOCOPHEROL

Chemical Information

I. Nomenclature

A. Common Names

1. Eta-tocopherol
2. 7-Methyltocol

B. Chemical Name

1. 2,7-Dimethyl-2(4',8',12'-trimethyltridecyl)-6-chromanol

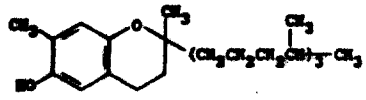
C. Trade Name

None

II. Empirical Formula

$C_{27}H_{46}O_2$

III. Structural Formula



IV. Molecular Weight

402.64

V. Specifications

Not available

VI. Description

A. General Characteristics

See alpha-tocopherol

B. Physical properties

Eta-tocopherol shows solubilities similar to alpha-tocopherol. The absorption maximum in ethanol is 298 nm (E1%/1 cm 104.1).

C. Stability

Same as alpha-tocopherol

VII. Analytical Methods

See alpha-tocopherol

VIII. Occurrence

A. Plant

Isolated from rice

B. Animal

None

C. Synthetics

See alpha-tocopherol

D. Natural inorganic sources

None

ALPHA-TOCOPHEROL ACETATE

Chemical Information

I. Nomenclature

A. Common Names

1. Alpha-tocopherol acetate
2. Vitamin E acetate
3. Alpha-tocopheryl acetate

B. Chemical Names

1. 2,5,7,8-tetramethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol acetate

C. Trade Names

1. Fertilvit
2. Endo E Dompe
3. Gevex
4. Alfacol
5. Epsilan-M
6. E-Toplex
7. Evipherol
8. Econ
9. E-Ferol
10. Tocopherex
11. Tofaxin
12. Ephynal Acetate
13. Contopheron
14. Ecofrol
15. Tocophrin

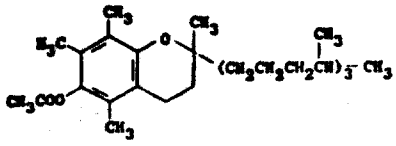
D. Chemical Abstracts Registry Numbers

1. Alpha Tocopherol Acetate - 000 133 802
2. D-Alpha Tocopherol Acetate - 000 058 957
3. D,L-Alpha Tocopherol Acetate - 000 054 228

II. Empirical Formula

$C_{31}H_{52}O_3$

III. Structural Formula



IV. Molecular Weight

472.73

V. Specifications

Food Chemicals Codex	
Assay	Not less than 97.0%
Refractive index	Between 1.494 and 1.498 at 20°
Limits of impurities	
Arsenic (as As)	Not more than 3 ppm
Heavy metals (as Pb)	Not more than 40 ppm
Lead	Not more than 10 ppm

VI. Description

A. General Characteristics

See alpha-tocopherol

B. Physical Properties

Alpha-tocopherol acetate is freely soluble in acetone, chloroform, and ether, is less readily soluble in alcohol and is practically insoluble in water. It exhibits the following characteristics: mp -27.5 degrees; $bp_{0.01}$ 184 degrees; $bp_{0.025}$ 194 degrees; $bp_{0.3}$ 224 degrees; d 21.3/4 0.9533; n 20/D 1.4950-1.4972; absorption maximum 285.5 nm (cyclohexane).

C. Stability

Alpha-tocopherol acetate is practically unaffected by the oxidizing influence of air, light, and ultraviolet light.

VII. Analytical Methods

The official method for the determination of alpha-tocopherol acetate involves the extraction, oxidative chromatography, saponification of alpha-tocopherol acetate and colorimetry of alpha-tocopherol (see alpha-tocopherol)

VIII. Occurrence

A. Plant

None

B. Animal

None

C. Synthetics

Alpha-tocopherol acetate is prepared from dl-alpha-tocopherol and acetic anhydride.

D. Natural inorganic sources

None

I. Acute toxicity

Substance	Animal	Sex & No.	Route	Dosage (mg/kg) Body Weight	Measurement	Ref.
Vitamin E Injectable	Mice	-	i.m.	gt* 250	LD_{50}	1761
Vitamin E Injectable	Rats	-	i.m.	gt 250	LD_{50}	1761
Vitamin E Injectable	Rats (neonates)	-	i.p.	gt 2000	LD_{50}	1761
Vitamin E Injectable	Rabbits	-	i.m.	gt . 50	LD_{50}	1761

*greater than

Demole reported that synthetic dl-alpha-tocopherol and its acetate were well tolerated by mice when orally administered in doses of 50 g/kg. Per os, frogs, rabbits, cats, dogs, and monkeys tolerated 0.2 g/kg of vitamin E without symptoms. The same dose was also tolerated without symptoms in frogs, rats, rabbits and dogs when administered subcutaneously, intramuscularly and intraperitoneally (572).

A male dog weighing 12.3 kg tolerated intramuscular doses of 2.5, 5.0, and 10 mg of vitamin E injectable/kg without symptoms. Hematology and clinical chemistries were normal (1761).

II. Short-term Studies

A. Tocopherols

Rats

Lehr et al. maintained a group of 15 albino rats (10-month-old) on a synthetic diet such that the average daily intake of alpha-tocopherol was 0.8 mg. With 18 rats, the experimental diet was enriched to yield a daily consumption of 10 mg alpha-tocopherol/rat. After 2 weeks, animals eating the enriched diet received supplementary stomach tube feedings of 10 mg alpha-tocopherol/100 g body weight 3 times weekly for 4 weeks. Following the third intubation, kidney damage was induced (by i.p. administration of sodium acetyl sulfathiazole) in 10 of the rats fed the unsupplemented synthetic diet and in 13 of the rats fed the enriched diet. All surviving animals were killed 62 days after renal blocking. Food intake was determined daily and the body weight checked twice weekly. Systolic blood pressure was recorded at predetermined intervals and estimations were made of N.P.N., calcium, and inorganic phosphates. All rats were subjected to complete postmortem examination. The heart, thymus gland, adrenals and kidneys were weighed immediately after removal. These organs as well as the aorta and gastrointestinal tract were examined microscopically. Alpha-tocopherol in excess was tolerated without noticable ill effects or histological alterations by all control rats except for the emergence of moderate hypertension. Disseminated muscular necrosis and hypertension following experimental nephropathy in rats maintained on complete

rations was not beneficially influenced by the presence of excessive amounts of alpha-tocopherol. Rather, the vitamin appeared responsible for a higher incidence of advanced lesions in aorta, coronary arteries and stomach (1463).

Groups of 5 male and 5 female rats (initial weight 80-100 g) were given d-alpha- and dl-alpha-tocopherol orally in daily doses of 200, 700, and 2000 mg/kg, 5 days per week for 3 months. The vitamin was administered by stomach tube as a suspension in an aqueous solution of cremophor. Records of body weight and general condition revealed no untoward effects. Blood picture, urinalysis, liver and kidney function tests, and blood chemistry were all normal. Histological examination of hearts, lungs, livers, kidneys, adrenals, spleens, stomachs, intestines, testes, ovaries, uteri and bone marrows disclosed no abnormalities. Thyroids showed slight hyperplasia at the highest dose (1761).

In a 98-day feeding study in which 2 groups of 10 male rats each were maintained on diets containing 12% supplements of either high or low potency alpha-tocopherol concentrates (25.5 and 13.7% tocopherols, respectively), the growth and food efficiency of the rats was found to be normal. It was concluded that the daily intake per rat on 460 mg of alpha-tocopherol was without effect (1761).

In an experiment in which groups of six 5-week-old male rats were maintained for 105 days on ground Purina Dog Chow alone or plus a supplement of 1 g/day (5 days per week) of deodorized refined cottonseed oil (Wesson Oil) or Alpha Tocopherol Concentrate (255 mg total tocopherols/g), no adverse effects resultant from the feeding of the experimental diets were observed during the experiment (1761).

For a period of 13 weeks, den Tonkelaar et al. maintained 2 groups of Wistar rats, 15 male and 15 female per group (initial body weight 40-60 g), on 15% lard diets containing 0.002 or 0.004% alpha-tocopherol. Ninety rats were maintained as controls; 60 of these received the 15% lard diets and the rest were maintained on a lard-free diet. After 13 weeks, 10 males and 10 females from the control lard group and 5 males and 5 females from each of the other groups were sacrificed. The liver, kidneys, heart, spleen, thymus, adrenals, thyroid, pituitary, pancreas, brain, uterus, ovaries, testes, and prostate were weighed and histopathological studies of the organs (all of those weighed, except the brain and thymus, plus the digestive tract and the bladder) of the rats of both control groups and of the group on 0.004% tocopherol were carried out. Blood was taken for hemoglobin estimation and erythrocyte and leucocyte counts. The protein content of the liver and the protein, water, and fat levels of the carcass were determined. The remaining animals were placed in individual cages on a fat-free diet having 20% of the caloric value of the normal mean daily food intake for male rats. Each animal received 3.5 g of food/day, and the animals were maintained on this diet until they died. The survival time of each rat was recorded and the same chemical determinations were carried out as on the sacrificed rats. After 9 weeks on the partial starvation diet, the surviving animals were fed the lard-free control diet ad libitum to detect any eventual difference in recovery between the groups. These animals were sacrificed 2 or 4 weeks later and hematological and

chemical analyses were carried out. Results of the experiment indicate no difference in growth or survival time. No difference in protein and fat utilization was found between the control groups and the groups which received alpha-tocopherol. Histopathological examinations and chemical determinations revealed no adverse effects attributable to the ingestion of alpha-tocopherol (2419).

In a study by Katsui and Iima in which 5 groups of young rats (2 male and 2 female, 30-40 g) received 0-50 mg of dl-alpha-tocopherol/rat/day for 40 days in a vitamin E deficient diet. The growth and appearance of all rats receiving tocopherol was normal. The growth rate of the vitamin treated rats was greater than the control group. The author estimated that the vitamin E requirement for rats was 1 mg per day or less (1265).

The administration of dl-alpha-tocopherol on 10 consectutive days was well tolerated by the male rat in the dose of a total of 1 g/kg orally. The normal female rat tolerated 0.5 g/kg without any symptoms. Histological investigation of the heart, lungs, liver, kidneys and testicles revealed no changes. The fertility of the rats remained unchanged (572).

Dl-alpha-tocopherol was administered for a period of two months orally to a group of 10 male and 10 female rats in a total dose of 4 g/kg. This dose was well tolerated. The estrus cycle of the rat, the fertility, the duration of the pregnancy, the number of offspring and their weight, and the milk secretion of the mother animals was unchanged. In comparison with the control animals, the testicles of the rats treated with vitamin E showed no significant changes in weight (572).

In a study by Demole, 12 normal white rats weighing 90-100 g were given daily, for a period of 49 days, 20 mg dl-alpha-tocopherol orally. All 12 rats increased normally in weight. They were paired, became pregnant and produced normal offspring. After 4 months they were killed and dissected and all were found to be healthy. None exhibited tumors (573).

Guinea pigs

Costa et al. administered orally 13 mg of alpha tocopherol per day to 10 guinea pigs for 10 days. In all of the treated animals an intense hyperemia of the thyroid was observed, especially in those capillaries and precapillaries which are found in direct contact with the follicles. No other morphological or color differences, as compared with the controls, were reported (487).

Prosperi and Borselli subjected 5 groups of guinea pigs (average weight 250-300 g) to intramuscular treatment with high doses of vitamin E (300 or 600 mg/day for 10 days; 600, 900, or 1200 mg in a single dose). The animals were sacrificed within 4 days after treatment and samples of

muscle, kidney, liver, adrenal, heart, and spleen were collected for histological examination. In the group treated with 100 mg/day for 10 days (Group I), the animals revealed a substantial increase in body weight (25-50 g), without any particular manifestations. The animals which received 300 mg/day for 10 days (Group II) demonstrated identical behavior, although the increase of body weight was only 10-30 g. The guinea pigs which received single doses of 600 (Group III) and 900 mg (Group IV) showed no signs of even minimal disturbance and their weights remained stationary. A single dose of 1200 mg (Group V) caused, immediately after the injection, signs of general pain, with roughness of the fur and little vivacity; the body weight remained invariable. Histological examination revealed a complete absence of lesions in Group I. In Group II there was a slight degree of ailment of the hepatic cell and the myocardial cell, with slight edema of the nervous substance. In the third group the hepatic cellular ailment is more outstanding, in the myocardium there is evidence of interfibrillary edema, and edema of the white cerebral matter is marked. Groups IV and V exhibited progressive development of these lesions and, in addition, in Group V notable hyperemia of the spleen was observed (1981).

Prosperi and Borselli also investigated the effect of large doses of vitamin E on the endocrine glands by treating, intramuscularly, 6 male and 6 female guinea pigs with 600 mg of Merck Evion/day for 3 days followed by 300 mg/day for 2 days. Three days after treatment the animals were sacrificed and samples of pituitary, thyroid, pancreas, adrenal, testicle, and ovary were subjected to histological examination. The results of the experiment were as follows: pituitary - intense acidophyle secretion with signs of initial functional exhaustion; thyroid - normal aspect, which presents on the whole alternate phases of activity; pancreas - greater number of islets, with prevalence of beta cells; adrenals - signs of ailment in the glomerular and reticular zones; testicle - the gland is in the terminal phase of an intense germinative activity; ovary - reveals the tendency toward an active maturative capacity of the numerous follicles in various stages of evolution (1981).

Man

In the treatment of lupus erythematosus, Welsh administered various daily dosages of vitamin E (natural mixed tocopherols and synthetic dl-alpha-tocopherol acetate), in conjunction with pantothenic acid derivatives, to 36 patients for periods ranging from 2 to 36 months. Eleven of the patients consumed over 200 g each in the course of the treatment. During the course of 20 months, one patient was given

424.2 g of vitamin E. In no case were any complications of side effects attributable to the ingestion of vitamin E observed (2564).

Hillman reported that the ingestion of 296 g of alpha-tocopherol (2 g daily for 37 days; 4 g daily for 55 days; and 2 g on the final day: in the form of Ephynal Acetate for the first 80 days and Aquasol E for the last 13 days) by a 41-year-old, normal man over a period of 93 days resulted in a sustained average plasma tocopherol concentration of 2.26 ± 0.86 mg per 100 ml. This was approximately twice the control level of 1.15 ± 0.37 mg per 100 ml. A significant transitory creatinuria occurred during the test period, but there was no apparent change in the excretion of creatinine or of 17-ketosteroids. There were no unequivocal signs of clinical toxicity. Exercise tolerance seemed unaffected. The electrocardiogram, ballistocardiogram, serum cholesterol, liver function and blood coagulation studies, and a muscle biopsy showed no deviation from the normal. However, chapping cheilosis and angular stomatitis appeared early and persisted variably, but without progression, throughout the ingestion period. Gastrointestinal disturbances were frequent, especially during the final month, when abdominal distress and diarrhea with tenesmus were pronounced. Vague generalized muscle weakness and increased fatigability were experienced toward the end of the test period. The untoward clinical manifestations disappeared within two weeks after the tocopherol was discontinued (1085).

B. Alpha-tocopherol acetate

Mice

For a period of 20 days, Ichihara injected ten 14-week-old male mice with 10 mg dl-alpha-tocopherol acetate daily. Following treatment, the animals were decapitated and their testes examined by electron microscope. Administration of the tocopherol caused enlargement of the testicular interstitial cells and marked development of the smooth endoplasmic reticulum, in particular the membrane whorls were more abundant in comparison with normal interstitial cells. On the basis of this data it was suggested that vitamin E administration activates the function of steroid hormone production in the testicular interstitial cells (1153).

Chicks

McCuaig and Motzok examined the effects of hypervitamosis E by feeding 2 groups of 9 day-old chicks (Vantress x White Plymouth Rock cockerels) for 32 days with a diet containing 1.0% dl-alpha-tocopherol acetate (10,000 I.U./kg of diet). Compared to chicks maintained on a normal diet, the treated chicks exhibited normal growth and livability. The livers of the high vitamin E animals were larger and darker red than those of the controls (1636).

For a period of 21 days, 9 groups of 20 chicks each (day-old White Mountain cross cockerels) were maintained on a control ration or the control ration supplemented with either 500 or 5000 I.U. per kg with either d-alpha-tocopheryl acetate or one of three commercial feed grade synthetic alpha-tocopheryl acetates (A, B, C). The chicks were weighed at 0, 7, 11, 14, 18 and 21 days. At termination of

the assay, feed consumption was determined, the chicks were sacrificed and examined for gross pathology, and the livers and kidneys were removed and weighed. There was no significant difference in growth between preparations at the 500 I.U./kg level. At the 5000 I.U./kg level, growth was significantly different between preparations with the group fed d-alpha-tocopheryl acetate showing the lowest gain (79% of control). No gross abnormalities were observed and kidney and liver weights showed no significant differences attributable to dose levels or preparations (1761).

Rats

For 25 weeks, Escudero and Herraiz maintained groups of vitamin E-depleted male rats (initial age 21 days) on diets containing supplements of alpha-tocopherol acetate such that the rats consumed 6-600 micrograms/day. The fertility of the animals was determined by 3 methods: 1. mating the animals during a period of 7 days with females of proven fertility and determining by autopsy if they were able to produce young; 2. determining the weight of the testicles of the animals in different groups; 3. determining by histological study the state of the testicles of the animals. The minimal dose of alpha-tocopherol acetate necessary to prevent sterility in the male rat was found to be 30 micrograms/day. The animals that received this dose showed little symptoms of testicular atrophy while those receiving smaller doses exhibited extensive testicular atrophy and 66% of those receiving 600 micrograms/day showed slight histological lesions of the testicles (723).

For periods of 4 to 6 weeks, Gray et al. maintained groups of 6 rats each on vitamin E-free diet supplemented with 0.5 (control group) or 100 mg of alpha-tocopherol acetate per day. At termination of the feeding period the animals were sacrificed, the livers were removed and weighed, and the liver DNA was determined. Growth of the treated animals was normal. An increase in liver weight, compared to the controls, was noted for the test rats. Determination of DNA revealed a significantly higher concentration of DNA in the liver of the rats fed excess vitamin E in comparison with the control animals. It was concluded that alpha-tocopherol acetate in excess amounts stimulates hyperplasia in rat liver in the young animal (932).

Seven groups of weanling male rats (from Blue Spruce Farms) were fed for 8 weeks either unsupplemented stock diet or the stock diet supplemented with 500 or 5000 I.U./kg of either d-alpha-tocopheryl acetate or either of two commercial feed grade synthetic alpha-tocopheryl acetates (A, B). All of the rats were observed and weighed weekly throughout the test period. After 8 weeks the animals were examined for gross pathology and the liver, kidneys, adrenals and testes were removed and weighed. Growth of all the treated rats was comparable to that of the control animals. No gross abnormalities were observed when the rats were autopsied and the weights of kidneys, testes, and adrenals were comparable for all groups of rats. The mean liver weight of rats fed both levels of the Commercial B alpha-tocopheryl acetate (4.80 g) was similar to that of the controls (4.85 g).

The mean liver weight of rats fed both levels of d-alpha-tocopheryl acetate was less (4.61 g) and that for the Commercial A synthetic alpha-tocopheryl acetate approached atrophy (4.45 g) (1761).

A test of the toxicity of d-alpha-tocopheryl acetate included an unsupplemented control group, a control group supplemented with 1.0 ml of olive oil per day and 4 experimental groups supplemented with 0.125, 0.25, 0.50 and 1.0 g of d-Alpha Tocopheryl Acetate Concentrate (200 mg tocopheryl acetate/g) per day. Six groups of 5 weanling female rats each were maintained on the toxicity experiment for 14 weeks. The supplements were administered orally by syringe 5 days per week (the 3 lower levels of tocopheryl acetate supplement were administered as dilutions in olive oil to provide a volume of 1.0 ml daily). The growth of rats receiving the d-Alpha Tocopheryl Acetate Concentrate at a level of 0.50 g/day and below was not significantly different from that of control rats. At the highest level of tocopheryl acetate (1.0 g/day), growth was depressed and 3 rats died within 2 weeks. Rats fed d-Alpha Tocopheryl Acetate Concentrate at levels of 0.50 g/day and below had feed efficiencies comparable to that of the olive oil supplemented group but lower than the unsupplemented control group. At the highest level of tocopheryl acetate supplementation (1 g/day), food consumption was greatly depressed (1761).

In an experiment in which groups of six 5-week-old male rats were maintained for 105 days on ground Purina Dog Chow alone or plus a supplement of 1 g/day (5 days per week) of deodorized refined cottonseed oil (Wesson Oil) or Alpha Tocopherol Acetate Concentrate (200 mg tocopherol acetate/g), no adverse effects resultant from the feeding of the experimental diets were observed during the experiment (1761).

DI-alpha-tocopherol acetate was administered for a period of two months orally to a group of 10 male and 10 female rats in a total dose of 4 g/kg. This dose was well tolerated. The extrus cycle of the rat, the fertility, the duration of the pregnancy, the number of offspring and their weight, and the milk secretion of the mother animals was unchanged. In comparison with the control animals, the testicles of the rats treated with vitamin E showed no significant changes in weight (572).

The administration of dl-alpha-tocopherol acetate on 10 consecutive days was well tolerated by the male rat in the dose of a total of 1 g/kg orally. The normal female rat tolerated 0.5 g/kg without any symptoms. Histological investigation of the heart, lungs, liver, kidneys and testicles revealed no changes. The fertility of the rats remained unchanged (572).

Rabbits

For a period of 1-3 months, Huter administered orally 5.5-12.5 mg tocopherol acetate/kg of body weight/day to 4 male and 6 female rabbits. In the female rabbits, highly active thyroids resulted, but with normal iodine protein contents, while in the males abnormal changes were observed. The thyroids of the male animals exhibited abnormal growth in volume and weight, absence of or deficiency in iodine and enhanced epithelial growth (1147).

Man

For a period of 9-10 days, 26 middle-aged healthy hospital patients received daily 400-500 mg of synthetic alpha-tocopherol acetate. This treatment tripled the blood tocopherol level. In 72% of the patients a distinct increase in thyroid uptake of iodine was observed and in 70% of the patients the level of serous organic iodine was increased slightly, indicating functional activation of the thyroid. A slight decrease in the basal consumption of oxygen was also observed in 58% of the patients (487).

In the treatment of 67 patients who had lupus erythematosus, Welsh administered, orally, one of the pantothenic acid derivatives in combination with one of the vitamin E preparations in massive doses according to the following schedule:

Pantothenic acid	
Calcium pantothenate	10-15 g daily
Pantothenyl alcohol	10-15 g daily
Sodium pantothenate	5-10 g daily
Vitamin E (alpha-tocopherol acetate)	
Ephynal acetate	1-2 g daily
Natopherol	1-2 g daily
Aquasol E	1-2 g daily

This program of therapy was maintained for intervals up to 3 years. There were no complications arising from this treatment program except for transient nausea and gastric distress in a few of the patients. Hematological studies and urinalyses revealed no abnormalities. Varying combinations and massive dosages of pantothenic acid derivatives and vitamin E preparations given to 154 patients who had diseases other than lupus erythematosus produced no complications except for gastric distress (2570).

Greenblatt gave 6 male subjects pure d-alpha-tocopherol acetate orally at a level of about 40 g/day for as long as a period of 1 month. No side reactions, weight gain, or loss occurred. A decrease in serum cholesterol was observed, with a rebound when the tocopherol treatment was terminated that almost reached pretreatment levels (946).

III. Long-term Studies

A. Tocopherols

None

B. Alpha-tocopherol acetate

None

IV. Special Studies

A. Tocopherols

Carcinogenic

In order to study the effects of hypervitaminosis E on lung tumor growth in mice, Telford maintained 200 young strain "A" (Bar Harbor hereditary lung tumor strain) mice, equally divided into 4 groups, on various diets for 7 months. Group 1 was maintained on a vitamin E-deficient diet. Group 2 received the deficient diet plus 2 mg of synthetic alpha-tocopherol on alternate days. Group 3 was held on a normal stock diet. Group 4 had normal diet plus 2 mg of alpha-tocopherol every other day. All animals received, s.c., 1 mg (1,2,5,6)dibenzanthracene in 0.25 ml olive oil. At the end of seven months all surviving animals were autopsied and the following results were obtained:

Group	*Diet*	*Number animals autopsied*	*Lung tumors*	*Cysts*	*Subc.-tumors*
			%	%	%
1	E-deficient	24	70.9	75.0	33.3
2	E-deficient plus α-tocopherol	37	94.6	24.3	35.1
3	Normal	22	90.9	22.7	36.4
4	Normal plus α-tocopherol	19	100.0	36.8	47.4

The incidence of lung tumor was greatest in the hypervitaminosis groups (2381).

For a period of 8 weeks, 10 adult male mice were smeared 3 times per week with a 10% dl-alpha-tocopherol solution in sesame oil on their depilated backs. In a similar manner 10 more mice were treated with a 1% benzopyrene solution in benzene. Just as on the untreated control animals, on the animals treated with tocopherol the hair cover reappeared as early as 8-10 days after the treatment. The skin of the tocopherol-treated mice remained supple and smooth without new formations of any kind. The mice treated with benzopyrene were incapable of replacing their hair cover and reacted after 2-3 weeks with a thickening of the skin on their backs and incipient callosity. Some of the benzopyrene-treated animals later developed warts and papilloma (573).

Two groups of 10 mice each were selected from fully grown female mice which, as a result of smearing the skin of their backs 3 times per week with a 1% benzopyrene solution in benzene, exhibited loss of hair, thickening of the epithelium, growth of warts, papilloma and carcinoma. All of the animals were further treated with benzopyrene and one half of them received in addition a daily dose of 10 mg dl-alpha-tocopherol per os. The mice were observed for a month for any variations in their condition. No significant difference was found between these animals which received alpha-tocopherol and those which didn't (573).

Demole injected 12 male adult albino rats subcutaneously in the back with 1 ml of a 1% oily benzopyrene solution. Four months later, at the start of sarcoma formation, the animals were divided into 2 groups. The rats in one of the groups received 0.2 ml of a 10% solution of dl-alpha-tocopherol in sesame oil 3 times per week per os (60 mg dl-alpha-tocopherol per week) for a period of 14-39 days. Growth of the sarcoma on the tocopherol-treated rats was approximately the same as for the untreated rats (573).

In a study by Shamberger and Rudolph, ICR Swiss female mice, 55-60 days old, were initiated once with 125 micrograms of 7,12-dimethylbenzanthracene dissolved in 0.25 ml of acetone. After a period of 3 weeks, the animals were painted 5 times weekly for 16 weeks with 0.25 ml of a mixture of 0.033% croton oil with or without 0.25% dl-alpha-tocopherol dissolved in an 80% acetone, 20% water solution. The tocopherol reduced the incidence of tumor formation in the mice (2192).

Kochur et al. reported that the administration of alpha-tocopherol or its acetate (7 i.p. injections of 3000 mg/kg each, in oil) to rats with various transplanted tumors retarded tumor growth and prolonged survival. Better results were obtained with emulsified alpha-tocopherol or alpha-tocopherol acetate (7 doses of 400 mg/kg each) prepared by stirring a 3:2 mixture in olive oil with an equal volume of isotonic NaCl solution containing 0.1% gelatin. No toxic effects of high doses of tocopherol were observed (1337).

Reproduction

To healthy, normally menstruating girls, Winkler administered, orally, 30 or 300 mg of alpha-tocopherol daily during the course of an estrus cycle. To determine the effect of this treatment on the estrus cycle, the urinary excretion of prolan, estrone and estriol was audited continuously. With doses of 30 mg alpha-tocopherol per day, an increase in hormone excretion was observed, while doses of 300 mg per day caused after 10 days, a reduction in hormone excretion. Disturbances of the rhythm of the estrus cycle, proiomenorrhea as well as opsomenorrhea, were also observed. According to Winkler, these effects were produced primarily through the action of alpha-tocopherol on the anterior lobe of the pituitary gland (2595).

In an effort to determine the effect of alpha-tocopherol on reproduction, 32 female rats were weaned during a 2-week period, were maintained on a vitamin E-free diet for 5 weeks, and then were transferred to a normal stock diet. When the rats had attained a weight of 150 g, they were mated over a period of 6 weeks. Insemination was determined by daily vaginal smearing. During the gestation period (21 days), d-alpha-tocopherol and dl-alpha-tocopherol were administered orally to the rats at levels of 100 and 500 mg/day. The total mean weight gained during the gestation period was comparable for both natural and synthetic tocopherol supplemented rats, the rats at the 500 mg/day levels producing slightly less weight gain than those at the lower level. It was suggested that this was probably due to the small mean litter sizes for these groups which was established prior to supplementation.

The length of the gestation was not effected by the tocopherol supplements. The mean weights of the young at 3 and 21 days were comparable for all groups. The viability of the tocopherol-supplemented rats was 80-90%. The young from tocopherol-supplemented rats were raised to weaning with practically 100% survival (1761).

The effect of tocopherol on resorption rate in the rat was examined by Telford et al. Walter Reed-Carworth Farms strain rats (200 g) in their first gestation were mated and then randomly distributed into experimental and control groups. Results of the study are presented in the following table (2384).

Treatment (per os)	Total Dose (gm)	Litters	Resorptions (%)
None (control)	-	126	10.6
dl-alpha-tocopherol acetate	0.2	12	4.1
d-gamma-tocopherol	0.2	11	4.7
d-gamma-tocopherol	0.07	12	5.7
dl-alpha-tocopherol acetate	0.1	14	14.0

In a study in which mature Broad Breasted Bronze turkey hens were maintained on an all vegetable protein diet for a period of 20 weeks, Ferguson et al. noted a slight increase in hatchability during the first 8 weeks of the experiment when dl-alpha-tocopherol was added to the diet (20 mg/lb) (770).

Sensitization

The delayed or tuberculin type of sensitizaton has been produced by Lipton in guinea pigs (as shown by skin test) using d-alpha-tocopherol incorporated into complete Freund's adjuvant. D-alpha-tocopherol and dl-alpha-tocopherol have also been shown to be sensitizing when injected into the skin in quantities of 0.01 and 0.02 ml. Dl-alpha-tocopherol acetate failed to produce sensitization (1496).

B. Alpha-tocopherol Acetate

Reproduction

Hill and Hamed investigated the possibility of hypervitaminosis-E in chicks by administering, parenterally, a total of 2000 mg of dl-alpha-tocopherol acetate per animal to 7 male and 7 female 3-week old chicks over a period of 10 weeks. Two male and 2 female chicks were maintained as controls. Body weight and, in the male, development of secondary sex characteristics were monitored. At termination of the test all animals were sacrificed and their testicles, ovaries, and other endocrine organs weighed and examined histologically. The animals treated with alpha-tocopherol acetate exhibited definitely inhibited growth, the inhibition intensifying in the course of the test period. While the male control animals showed all indications of beginning sexual maturity during the last weeks of the test period, increasingly evident symptoms of debility, lack of appetite as well as dull plumage and an inclination to moult were detectable in the animals

treated with vitamin E. The crest, wattles, and testicles of the treated roosters remained considerable, under-developed and histological examination of the testicles gave no indications whatsoever of sexual maturity. However, the ovary weights and the histological examinations of the ovaries of the treated females revealed no changes in this organ as compared to those of the control animals. Histological examination and weights of the other endocrine glands also indicated no changes in these organs (1083).

In a study by Czyba, eleven male adult hamsters (about 6 months old) were given daily, by subcutaneous injection, 75 mg of dl-alpha-tocopherol acetate. Four other hamsters were maintained as controls. The animals were sacrificed after receiving treatment for 8-30 days. The testicles were weighed and, along with the epididymis and seminal vesicles, subjected to histological examination. In all cases the weight of the testicles of the treated animals was found to be significantly lower than the corresponding value for the control animals. Histological examination showed that spermatogenesis occurred normally in the control animals but that it was more or less disrupted in the treated males. These effects seem to be transitional, since after a delay of about 3 weeks the spermatogenesis was restored. The endocrine function of the testicles remained unaffected throughout the test period (506).

Following a feeding study which included an unsupplemented control group, a control group supplemented with 1.0 ml of olive oil per day and experimental groups (5 weanling female rats per group) supplemented with 0.125 and 0.25 g of d-Alpha Tocopheryl Acetate Concentrate (200 mg tocopheryl acetate/g) per day which were given these supplements 5 days/week for 14 weeks, these rats were mated to determine the effect of tocopheryl acetate on reproduction. Supplements of tocopheryl acetate were continued until all control rats had delivered their young. Results of the reproduction study indicate that d-Alpha Tocopheryl Acetate Concentrate did not inhibit fertility or number of young born per litter. The viability of young from rats given tocopheryl acetate appeared to be better than that for young from control rats (1761).

BIOCHEMICAL ASPECTS

I. Breakdown

Qualfe et al. examined the loss of vitamin E in foods due to cooking. Deep fat frying was found to be very destructive; for example, losses of tocopherol in doughnut fat range from 70 to 90%. Potato chips contain 0.1 mg tocopherol/g of extracted fat, compared to the usual concentration of tocopherol in vegetable oils of about 1 mg/g. Baking apparently destroys tocopherol to a lesser extent; extracted fat from a series of pies, cakes, and cookies had 0.26-0.76 mg tocopherol/g (2000).

Booth and Bradford found that the immersion of vegetables in boiling water for 30 minutes reduced the alpha-tocopherol content of the vegetables by an average of 3.7% (271).

Frazer and Lines reported that the aerobic storage of freshly milled flour for 190 days resulted in a 62-67% reduction of the tocopherol level (808).

Bunnell et al. reported that while ordinary cooking processes used in preparing food for the table do not involve very large losses of tocopherol, commercial processing and storage result in a considerable loss. Cold-temperature storage has been found not to prevent the loss of tocopherol (340).

II. Absorption-Distribution

Pearson and Barnes injected 600 to 800 micrograms, alpha-, beta-, gamma-, and delta-tocopherol in 0.2 ml glycerol trioleate into the small intestine loops of hooded female rats to study the relative absorption of the compounds. The tocopherols were absorbed from the intestinal loops in the following order and in the following per cents of the total administered doses (within 6 hours) (1902):

1.	alpha	32%
2.	gamma	30%
3.	beta	18%
4.	delta	1.8%

By administration of oral doses of DL(5-Me-^{3}H)alpha-tocopherol (with 0.2 mg of carrier alpha-tocopherol) to 50 fasting subjects with various disease states, with or without steatorrhea, Kelleher and Losowsky investigated the absorption of alpha-tocopherol in man. The average absorption of alpha-tocopherol in subjects without steatorrhea amounted to 72.4% of the administered dose while only 59.4% was absorbed by subjects exhibiting steatorrhea. The peak of plasma radioactivity occurred 6-12 hours after oral administration. Thin-layer chromatography of plasma extracts showed that more than 80% of the radioactivity in plasma was present as unchanged alpha-tocopherol. The remaining radioactivity was mainly alpha-tocopherolquinone. Urinary excretion of the radioactivity was almost always less than 6% (1283).

In a test of tocopherol absorption in man, by Schmandke et al., following the oral administration of 10, 30, 50, 100, 500, 1500, and 2000 mg dl-alpha-tocopherol to male test subjects ranging in age from 18 to 34 years, the corresponding absorption rates amounted to 96.9 ± 13.0%, 87.3 ± 13.5%, 72.6 ± 11.6%, 81.5 ± 6.1%, 67.6 ± 19.4%, 70.3 ± 27.9%, and 55.2 ± 28.8% (2136).

MacMahon and Neale administered oral doses of 12 to 25 microcuries tritiated alpha-tocopherol in 1 mg unlabelled alpha-tocopherol in propylene glycol to 7 fasting humans. The 7 subjects had normal plasma levels of tocopherol of 614 to 1151 micrograms/100 ml. It was found that they absorbed 55.0 to 78.6% of the dose. The urine recovery was 3.4 to 16.1% of the dose in 3 days, which was related to the amount absorbed. The plasma recovery began in 1 to 3 hours, peaked at 5 to 9 hours, and had a mean half-life of 53 hours. The plasma radioactivity recovered was found to be 90% free alpha-tocopherol (vitamin E). The vitamin E absorption was also studied in 23 patients having intestinal malabsorptions of at least 4 types. Patients having biliary obstructions exhibited the worst malabsorption of vitamin E suggesting that bile salts may be needed for the absorption of vitamin E. Patients having intestinal mucosal problems absorbed only small amounts of vitamin E. A good correlation between steatorrhoea and malabsorption of vitamin E was shown. Since the patients with intestinal lymphangiectasia were equally as unable to absorb vitamin E, the lymphatic pathway appeared to be significant in the absorption of vitamin E, which is fat-soluble. Mild vitamin E malabsorption was found in only 5 out of 6 patients with partial gastrectomy, although vitamin E deficiency often is found in such cases. The authors suggested that no conclusions could be made about whether dietary vitamin E is as well absorbed as free alpha-tocopherol (1532).

The investigations of Gallo-Torres, using normal female rats subjected to cannulation of the abdominal thoracic duct, indicate that bile is essential for the intestinal absorption of dl-alpha-tocopherol acetate and that pancreatic juice seems to be necessary for the hydrolysis of the vitamin E acetate ester (837).

Weber et al. found that the gastrointestinal absorption of d- and l-alpha-tocopherol following the oral administration to rats is considerably higher (about 55%) when the two compounds are administered in a 1:1 ratio than when the compounds are administered individually. When the tocopherols are administered as a mixture the two compounds are absorbed to nearly the same extent (2546).

On the basis of experiments in which fasted rats received, d-alpha- and l-alpha-tocopherol acetate orally, Weber et al. concluded that l-alpha-tocopherol is absorbed better and excreted somewhat more rapidly than the d-form (2550).

By administering 2 g of vitamin E, as alpha-tocopherol or alpha-tocopherol acetate, to 26 normal human subjects, Gounelle et al. found that there results a hypervitaminemia E which peaks in the sixth hour after administration. The average percentage increase following administration of alpha-tocopherol was 118% while that following alpha-tocopherol acetate was 36.3%, thus indicating that alpha-tocopherol is much more readily absorbed as the free form than as the acetate ester (909).

In vitro and in vivo studies by Peake and Bieri revealed that rat tissues take up alpha- and gamma- tocopherol to approximately the same extent, and in red blood cells a slight preference for gamma-tocopherol exists. Tissues, however, showed a more rapid disappearance of gamma-tocopherol. Thin-layer chromatographic separation of alpha- and gamma-tocopherol in heart, kidney, testes, and muscle extracts showed that 75-85% of radioactivity was present as unchanged tocopherol at all times (1900).

On the basis of studies using lymph duct cannulated rats, MacMahon et al. stated that under normal circumstances absorption of alpha-tocopherol occurs mainly via the lymphatic pathway. Studies with bile duct cannulated rats suggest that small amounts (8%) of alpha-tocopherol can be transported from the intestine via the portal vein (1533).

Johnson and Pover conducted an investigation into the pathway of absorption of ^{14}C-labelled alpha-tocopherol. Rats with cannulated intestinal lymphatic ducts were given single oral doses of 60 micrograms of labelled alpha-tocopherol (1 mc/mM). The resultant radioactivity of the chyle and urine were determined. The results suggest that the primary absorption pathway of alpha-tocopherol is via the intestinal lacteals into the chyle. Chromatography of the chyle lipids indicated that alpha-tocopherol passes unchanged into the lymphatic system (1218).

Blomstrand and Forsgren studied tocopherol absorption by administering oral doses (in physiologic proportions) of various labelled tocopherols to 2 female hospital patients with cannulated thoracic ducts. The total recovery of the administered radioactivity of tocopherol and its derivatives in the lymph lipids ranged from 21.3 to 90,9% in 24 hours, absorption into the lymph occurring mainly between 2 and 8 hours after administration. Most of the radioactivity was found in the chylomicrons. In general, the tocopherols exhibited a low absorption rate, dl-alpha-tocopherol acetate exhibiting greater absorption than dl-alpha-tocopherol. When tocopherol acetate was administered it was recovered as tocopherol acetate. The authors concluded that tocopherols are absorbed via the lymphatic pathway in man; tocopherol enters the thoracic duct intact and tocopherol acetate is split (255).

To determine the relative rates of liver storage of alpha-, beta-, gamma- and delta-tocopherols in the growing chick, Griffiths administered these compounds, orally, to vitamin E-depleted chicks over a period of 5 days. Two days after the final dose the birds were killed and chromatographic determinations produced the following results (949).

	Total dose 6 birds (mgms.)	Concentration found in fresh liver (µgm./gm.)	Total recovered in livers (mgm.)	Relative percentage recovery (α = 100)
α-Tocopherol	120	1·00 ± 0·19	0·280	100
β-Tocopherol	100	0·76 ± 0·17	0·090	41·2
γ-Tocopherol	120	0·34 ± 0·15	0·054	28·7
δ-Tocopherol	100	Nil	Nil	Nil

In dose levels approximately a reasonable dietary intake, alpha-tocopherol-C^{14} was administered orally, by Krishnamurthy and Bieri, to rats and chicks and its distribution in the body and rate of excretion were determined at intervals up to 21 days. The radioactivity in all tissues was identified almost exclusively as unchanged alpha-tocopherol except after 21 days, when trace amounts of unknown compounds appeared in chick liver and kidney. The distribution of radioactivity in the rat following a single oral dose of tocopherol is shown in Table 2 and the rate of depletion therefrom is indicated in Figure 1. No evidence was obtained for a significant accumulation of tocopheryl quinone or other metabolic products in either rat or chick tissue. It was suggested that the lack of metabolites was related to the low dose of tocopherol administered. Alpha-tocopherol in rat liver and intestinal mucosa cells was distributed about 50-60% in the mitochondria and 15-20% in the microsomal and supernatant fractions (1375).

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN THE TISSUES OF RAT AFTER A SINGLE ORAL DOSE OF 500 μc OF α-TOCOPHEROL-C^{14} (500,000 cpm)*

Tissue	% of Administered Dose in Whole Organ or Tissue		
	Hours After Dose		
	2.5	4.5	24†
Liver	6.40	5.40	5.53
Heart	0.06	0.06	0.37
Plasma‡	1.12	2.40	1.70
Erythrocytes	0.40	0.31	1.56
Small intestine	11.00	8.00	4.06
Kidneys	0.57	1.50	0.90
Brain	0.08	0.05	0.20
Spleen	0.11	0.14	1.16
Stomach	0.59	0.15	0.41
Lungs	0.15	0.32	0.84
Mesenteric fat (per g)§	0.12	0.31	0.88
Intestinal and stomach contents	27.0	8.12	1.05
Cecal contents and feces	6.80	28.20	39.82
Total	54.40	54.99	58.48

* Values are averages of results in two rats.

† Rats fitted with tail cups to prevent coprophagy.

‡ Total value calculated on the assumption that the rat contains 6% of its weight as blood.

§ This value expressed as percentage of dose per gram of tissue since no estimate of total mass of mesenteric fat was made.

In an effort to trace the route of alpha-tocopherol in rats, Mellors and Barnes administered by stomach tube 0.5 ml of a solution of (5-Me-^{14}C)-alpha-tocopherol in arachis oil (0.36 microcuries/ml) to 12 male rats, 4-6 months old. The distribution of radioactivity in rat tissues 3, 6, 12 and 24 hours after dosing is presented in the following table.

	Radioactivity											
	Counts/min g tissue				Counts/min g lipid				% of dose			
Tissue	3 h	6 h	12 h	24 h	3 h	6 h	12 h	24 h	3 h	6 h	12 h	24 h
Liver	260	885	482	810	6850	22200	16300	9580	1·96	4·20	2·80	3·74
Kidney	37	448	105	190	69	9170	4430	5120	0·05	0·39	0·11	0·17
'Stomach'*	3100	2040	305	126	60000	36200	10900	2290	13·07	3·30	1·05	0·29
'Intestine'*	2920	963	467	346	17080	7060	2340	1600	16·24	3·75	2·71	1·67
'Caecum'*	405	5170	1263	551	7720	95500	14450	6390	2·17	20·28	5·43	2·00
Testis	7	134	68	91	299	3720	2520	2830	0·00	0·15	0·09	0·10
Spleen	387	986	1110	1271	13640	46200	27500	28700	0·09	0·25	0·25	0·19
Adrenal	543	1510	5380	1675	4550	6970	38600	22280	0·01	0·03	0·11	0·03
Pelt	110	85	103	166	1875	685	995	1920	2·72	1·58	2·50	3·52
Rest of carcass	392	93	216	148	3820	1569	1935	1225	30·40	7·48	15·55	8·22
								Total recovery	66·71	41·41	30·60	19·93

* Includes contents at time of killing.

Intracellular distribution of the radioactivity found in rat liver and spleen was as follows:

	Radioactivity			Radioactivity	
Tissue	Counts/min	% total	Tissue	Counts/min	% total
Spleen:			Liver:		
Nuclei	1846	9·6	Nuclei	330	13·5
Mitochondria	11325	59·3	Mitochondria	300	12·2
Microsomes	3601	18·8	Microsomes	1070	43·7
Supernatant fraction	2334	12·2	Supernatant fraction	735	30·6

Absorbed alpha-tocopherol and its metabolites appeared not to re-enter the lumen of the gut in significant amount through the bile or by the secretion from the stomach mucosal cells within the 24 hour experimental period. Analysis of liver and spleen lipids showed that most of the radioactivity in the lipids was due to the presence of unchanged alpha-tocopherol. A much smaller amount of radioactive tocopherolquinone was isolated from both spleen and liver lipids and there was some evidence, in the liver lipids, that a second oxidation product of alpha-tocopherol, compound O, had been formed (1655).

Gloor et al. investigated the distribution of alpha-tocopherol in the rat by administering daily oral doses of 1 mg ^{14}C-dl-alpha-tocopherol acetate to vitamin E depleted rats for 5 days. The radioactivity of the rat tissues was measured up to 88 days after administration. The results of this study are presented in Table 3 (888).

Table 3. Alpha-tocopherol content in micrograms/g of fresh organ weight in rats after 5 doses of 1 mg dl-alpha-tocopherol acetate each.

Organ	4 days	11 days	18 days	32 days	47 days	68 days	88 days
Blood	4.2	1.6	1.1	0.6	0.2	-	0.1
Liver	14.4	6.7	3.1	1.7	0.6	0.7	0.4
Muscle	6.4	3.8	2.8	1.6	0.9	0.7	0.2
Kidney	13.0	6.3	3.8	2.1	0.9	0.7	0.2
Adrenal	97.0	52.5	25.6	11.6	4.1	4.0	2.3
Pituitary	34.2	15.3	11.4	5.8	8.1	3.4	1.5
Heart	17.8	10.2	6.3	4.3	1.5	1.4	0.5
Testes	8.4	4.6	3.5	3.3	1.7	2.3	1.0
Brain	5.5	4.0	4.4	4.0	2.9	3.1	3.1
Skin	10.5	4.5	3.2	1.1	0.5	1.3	0.4
Fat	12.5	14.5	7.0	3.2	1.7	2.3	0.8

In a study by McMasters et al., 19 hospitalized male patients, 30 to 81 years of age, were given 1 g of mixed tocopherols (60% d-alpha-tocopherol) orally, every day for two weeks. Tocopherol levels in plasma and adipose tissue were determined at the start and immediately after the last supplement was taken. Average plasma tocopherol levels increased from 3.7 to 11.3 micrograms/ml and adipose tocopherol levels increased from 52 to 119 micrograms/g (1647).

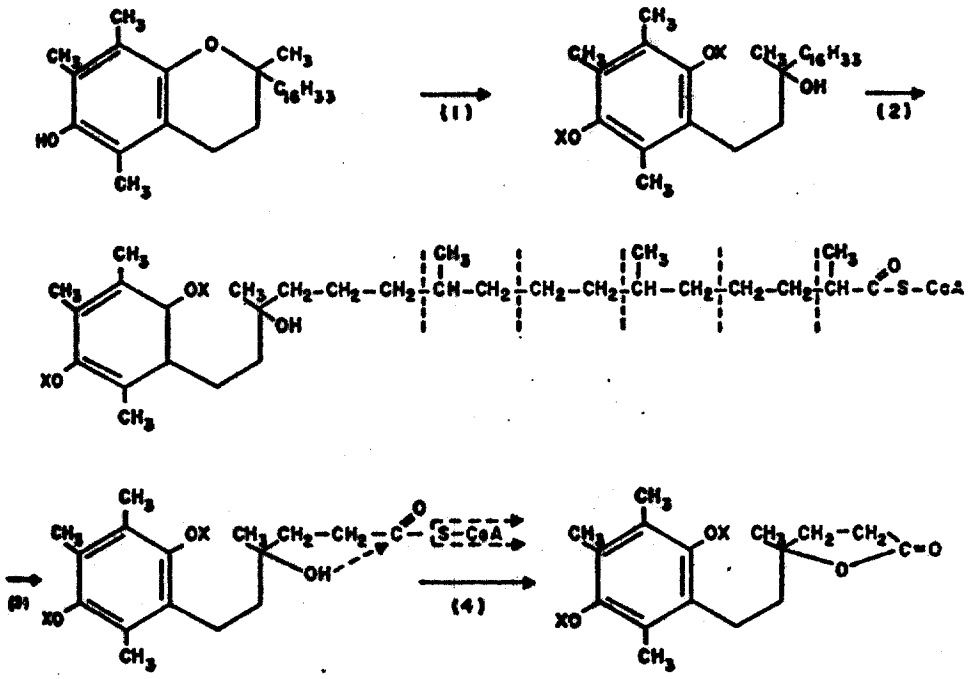
Following the oral administration of tocopherol to 18 normal, healthy male subjects (30-40 years old) at a level of 1.0 g/day for 2 weeks, McMasters et al. noted in increase in the tocopherol levels of adipose tissue from 38 to 80 micrograms/g and of plasma from 4 to 11 micrograms/ml. The levels declined rapidly when supplementation ceased; 2 weeks after supplementation had ended the average tocopherol level of adipose tissue was 54 micrograms/g and that for plasma was 7 micrograms/g (1648).

III. Metabolism and Excretion

Kaludin gave rabbits a mixture of tocopherols per os - 60 mg/animal (each rabbit weighed an average of 3 kg) of 43% alpha-, 45% beta-, 45% gamma-, and 12% delta-tocopherol. After an absorption period of 24 hours, the muscles, liver, heart, kidneys, and urine contained only alpha-tocopherol. The untreated controls also had only alpha-tocopherol in the organs studied. The author assumed that since only alpha-tocopherol was excreted, the beta-, gamma-, and delta- compounds must have been converted. Two unidentified fractions were observed in the urine, blood, and organ chromatograms of test and control rabbits, which may represent intermediate products in tocopherol metabolism. The blood of control rabbits contained only alpha-tocopherol; the blood of treated rabbits contained all 4 tocopherols after 6 hours. The beta-, gamma-, and delta- portions were found in only trace amounts after 24 hours (1244).

To determine if hens are capable of converting gamma- or delta-tocopherol to alpha-tocopherol, Dju et al. maintained laying hens on a vitamin E-free diet supplemented with pure alpha-, gamma-, or delta-tocopherol at levels of 100-4000 mg/week. Analysis of their eggs for total and for gamma- and delta-tocopherol content provided no evidence of conversion of gamma- or delta-tocopherol to alpha-tocopherol (632).

Following daily oral doses of 3-5 g of dl-alpha-tocopherol to healthy subjects, Simon et al. collected the urine in 24-hour samples. As in a previous study using rabbits (2241), analysis of the urine revealed the presence of two metabolites of dl-alpha-tocopherol, largely in conjugated form. Chemical, spectroscopic, and analytical evidence was presented for the formulation of their chemical structures as 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone and its gamma-lactone. A hypothetical scheme for the biological formation of the metabolites is shown in Fig. 0. In the scheme the chroman ring is opened hydrolytically, and the hydroquinone is conjugated (Step 1). The terminal methyl group of the side chain is oxidized to a carboxyl group, and the resulting fatty acid is conjugated with coenzyme A (Step 2) and degraded by beta-oxidation (Step 3). The termination of beta-oxidation 2 carbons short of completion may be explained by a nucleophilic attack by the hydroxy group on the acyl coenzyme A to form the stable gamma-lactone, pushing out the coenzyme A molecule and thus terminating beta-oxidation (Step 4) (2242).



Hypothetical scheme for the biological formation of the metabolites. X may be a hydrogen atom or a glucuronic acid residue.

Following the oral administration of 0.5, 1.0, 1.5, and 2.0 g of dl-alpha-tocopherol to healthy male test subjects, Schmandke recovered from the urine 0.96, 1.91, 0.82, and 0.72%, respectively, of the compound in the form of tocopheronolactone (2130).

After the oral administration of 1 g dl-alpha-tocopherol to healthy men, Schmandke and Schmidt found that about 40% is absorbed and that 6% of the absorbed tocopherol is reduced to tocopheronolactone; of 1 g of orally administered dl-alpha-tocoquinone, about 75% is absorbed and 1.8% of that absorbed is reduced to tocopheronolactone. On the basis of the low rate of change of dl-alpha-tocoquinone to tocopheronolactone in comparison with dl-alpha-tocopherol, the authors suggested that the mechanism of alpha-tocopherol catabolism by way of alpha-tocoquinone to tocopheronolactone plays only a small part in the catabolism of alpha-tocopherol to tocopheronolactone in the human organism (2131).

Alaupovic et al. have demonstrated that alpha-tocopherol-5-methyl-C^{14} is converted in rats and pigs, following intraperitoneal injection, into 3 metabolites: compound F and compounds Oi and Os. These compounds are oils, and they were isolated and separated from liver extracts by a combination of column and paper chromatographic methods. Compound F, which is the main metabolite, can be reduced readily to an Emmerie-Engel-positive material. Neither compound is a quinone or hydroquinone, and neither contains phosphorus in its molecule. It has been shown that the compounds alpha-tocopherylquinone, alpha-tocopheryl-hydroquinone, alpha-tocopheroxide, trimethylphytylbenzoquinone and Simon's metabolites, which have been suggested as possible metabolic or degradation products of alpha-tocopherol, are not present in rat and pig liver under conditions used in these experiments (29).

IV. Effects on Enzymes and Other Biochemical Parameters

The concentrations of ATP, ADP, and CoA in the liver of male albino rats were found, by Gray et al., to be significantly elevated by the addition of 100 mg of alpha-tocopherol acetate daily to the diet of the rats for 20 days. To explain this phenomenon the following scheme was proposed (931):

$$(1)\ AH_2 + B + CoA \rightleftarrows A \sim SCoA + BH_2$$
$$(2)\ A \sim SCoA + \alpha\text{-}T \rightleftarrows A + CoAS \sim \alpha\text{-}T$$
$$(3)\ CoAS \sim \alpha\text{-}T + Pi \rightleftarrows CoAS \sim P + \alpha\text{-}T$$
$$(4)\ CoAS \sim P + ADP \rightleftarrows CoA + ATP$$
$$(5)\ Dephospho\text{-}CoA + ATP \rightleftarrows CoA + ADP$$

D'Agostino noted that healthy young rats showed reduced vitamin B, in their urine after the daily administration of 150-600 mg of alpha-tocopherol for 1-4 days. Similar results were obtained by simultaneous administration of vitamin B, and alpha-tocopherol (512).

In experiments conducted by Smolins'ka it was found that oral administration of 25, 50, or 100 mg of vitamin E daily to rats results

in an increase of copper and cobalt concentrations in the liver, muscles, and bones. The effect of vitamin E on iron content of the organs and tissues was found to be dose-dependent. Doses of 25 mg daily cause a decrease of iron content in the liver and muscles and an increase in the bones; 50 mg per day cause a decrease of iron content in the liver and an increase in the muscles and bones; 100 mg per day decreases the iron content in the bones and increases its concentration in the muscles and liver (2131).

Gray and Loh noted significant increases in phospholipids, total cholesterol, and alpha-2 globulins and significant decreases in amino acids in the plasma of healthy medical students following daily ingestion of 100 mg of alpha-tocopherol acetate for 10 days (930).

Gray found that male albino rats maintained for 8 days on a standard diet, with 100 mg alpha-tocopherol acetate per rat added as daily dietary supplement, had signifantly higher levels of liver phospholipids, total and ester cholesterol than their controls. Liver homogenates from these rats synthesized, with glycerol as substrate, significantly greater amounts of these lipids than the control homogenates. Gray suggested that these changes indicate that the liver cell itself is in some way altered by the tocopherol (926).

Marx et al. found that hypervitaminosis E had no effect on the blood or tissue cholesterol levels of the rat. However, high doses of a vitamin E concentrate (51 mg of "mixed tocopherols" per week) produced localized sclerosis and deposition of cholesterol in the aorta (1592).

The coagulation time and prothrombin time of blood taken from 23 human subjects before and 1, 2, and 3 hours after the oral administration of a single dose (100 mg) of vitamin E (Ephinal) was determined by Prosperi. In all cases, at 1 hour after administration the coagulation time was reduced 20-80%. In 7 cases the second hour showed lowest values and at the third hour all but 7 had returned to normal. Quicks prothrombin time was lower in 13 of 23 cases more than 4 seconds. Similar results were produced by in vitro tests (1977).

With the administration of alpha-tocopherol (100 mg daily for 7 days orally, 50 mg for 10 days i.m., or 100 mg i.v. once) to a total of 15 men, Feruglio and Petrin observed a reduction of the clotting time, an increase of thromboplastin activity and a sharp increase of plasma antithrombin, the total effect being a trend toward hypercoagulation (775).

Byshevskiy reported than an excess of vitamin E (0.15 mg/animal/day) in the composition of a ration leading to the development of hypercholesterolemia (containing 3 mg of methylthiouracil/day and 0.2 g of cholesterol/day) in rats increased the depression of the anticoagulative system which was observed when the ration was fed with a normal level of vitamin E (371).

Tul'chinskaya and Mishnaevskiy reported that the feeding of vitamin E to young chicks increased the output of normal antigen agglutinins and the titer of blood complement, and stimulated the phagocytic activity of the leukocytes (2438).

March et al. reported that the addition of dl-alpha-tocopherol acetate (120 IU/kg) to the normal, balanced diet of chicks resulted in an increase of the number of circulating reticulocytes. Hematocrit values and erythrocyte lifespans remained unaffected. The reticulocytosis is attributed to retardation of the maturation process of the cells rather than to acceleration of erythropoiesis (1559).

Rave et al., through both in vivo and in vitro tests, found that the administration of vitamin E (Evion-Aquat) to rats inhibits the intensity of mesenchyme metabolism. This effect was found to be dose-dependent and to vary in different organs (2018).

Czyba reported that in the castrated female golden hamster alpha-tocopherol exerts an estro-progesterone effect when administered at a dose of 10 mg daily. When the daily dose is increased to 100 mg the estrogen effect becomes predominant (505).

Ichihara studied the anterior pituitary from mature virgin female, pregnant, and vitamin E-treated (10 mg/day for 11-18 days, injected) pregnant mice by means of an electron microscope. It was found that during gestation both rough endoplasmic reticulum and the Golgi region developed well in GH cells, LTH cells and LH cells in comparison with normal mature females. The development of both organelles was greater in the vitamin E-treated mice. It was concluded that the administration of vitamin E to pregnant mice activates the secretory function of GH, LTH, and LH in the anterior pituitary (1152).

In normal rats and guinea pigs, Chauchard found that small doses of vitamin E, i.p., exerted first an excitant and then a depressant action on chronaxia, acting on the brain, medulla and spinal cord (431).

Baroni and Casa found that alpha-tocopherol (300 mg parentally, single dose; 300 mg/day orally plus 300 mg/day i.m. for one week; 300 mg every other day for 30 days, i.m.) lowers the basal metabolic rate in man, as determined by oxygen uptake. Prolonged administration increases the effect (125).

Becker-Freyseng reported that, in patients with oliguria due to diencephalic hypophysis, 30 mg of alpha-tocopherol acetate (single injection) increases the diuresis while 60 mg of the tocopherol exhibits an inhibiting influence on the same (156).

Estrus was induced, by Szarka, in 19 of 29 infantile rats (3-5 weeks old) by the feeding or subcutaneous injection of substances high in vitamin E. The vitamin was prepared from wheat embryo and used in the form of an ethereal extract, as an unsaponifiable fraction or as an unsaponifiable fraction free of sitostearin. All 13 control animals remained negative (2347).

V. Drug Interation

Swick and Baumann found that administration of high doses of alpha-tocopherol, gamma-tocopherol, or alpha-tocopherol acetate dminished the storage of vitamin A in the livers and kidneys of rats fed moderate

amounts of beta-carotene. This effect was not observed when preformed vitamin A was fed (2340).

In bio-assays of vitamin A in rats, alpha-tocopherol, but not alpha-tocopherol acetate, increased the rate of gain in the assay period. In a post-assay depletion period, animals that received alpha-tocopherol during the assay period survived 30-80% longer than controls. As judged by gain in weight, there is an optimum dosage of alpha-tocopherol (between 0.5 and 1.5 mg/day/rat) for conservation of vitamin A (1685).

Maddock et al. reported that long-continued ingestion of moderate doses of vitamin A (50-250 IU/g body weight/day) in weanling rats elicits degenerative lesions of the testis, but in mature rats receiving similar dosages these lesions do not develop. Vitamin E (alpha-tocopherol acetate, 10-100 mg/50 g body weight/day) given concurrently with vitamin A potentiates the degenerative change (1535).

Kryukova and Pechenina noted that the introduction of 20,000 IU of vitamin A daily into rats per os decreases the oxidative phosphorylation P/O coefficient in rat liver mitochondria. The additional administration of 50 mg of tocopherol daily to the rats further disrupted the process of oxidative phosphorylation (1385).

In a 5-week feeding study, Dattatrey Rao et al. verified the vitamin A-sparing activity of alpha-tocopherol (537).

In a feeding study employing day-old chicks, McCuaig and Motzok showed that the simultaneous feeding of large amounts of dl-alpha-tocopheryl acetate (1.0% of the diet) with vitamin A prevented the adverse effects of hypervitaminosis A (1636).

Gazet du Chatelier reported that dl-alpha-tocopherol administered at high dosage (50 mg/kg) sensitized the guinea pig to the action of histamine, uniformly causing death in the second intoxication. At lower dosages no sensitization was observed (848).

In dogs treated with vitamin E (100 mg/day for 4 days, i.p.), Luise found the amount of barbiturate (pentothol or farmotol) necessary to produce profound narcosis is less than that required for the untreated controls. In order to maintain a state of narcosis in animals treated with vitamin E it is necessary to administer larger doses of barbiturates than are required by the controls. The duration of the narcosis produced by equal amounts of barbiturate is less in animals treated with vitamin E than in the untreated controls (1518).

Uskov reported that doses of 0.5 g/kg alpha-tocopherol acetate decreases the mortality rate of mice from phenamine (14 mg/kg), while doses of 1 and 2 g/kg were ineffective. He also found that doses of 1 g/kg alpha-tocopherol acetate distinctly strengthen the effect of narcotics (hexobarbital, Trapanal, ethyl alcohol) and analgesics (morphine, Promedol, Phenadone) on the central nervous system, without raising their toxicity (2437).

A further study by Uskov showed that alpha-tocopherol acetate in doses of 0.5 g/kg increased the effect of semicarbazide and Tubazid in mice

while a dose of 1.0 g/kg exhibited a protective action against the effects of Corazole (2438).

Harber and Wissler found that increasing the dietary intake of alpha-tocopherol 100-fold or more above the normal intake markedly reduced the incidence of tumor in 57 mice injected with methylcholanthrene (984).

Gallagher found that the prior or simultaneous administration of alpha-tocopherol acetate (95 IU) afforded protection to rats given lethal doses of carbon tetrachloride, presumably through its antioxidant activity (836).

Arscott et al. reported that the addition of vitamin E (0.5 g of Myvamix; 44 IU/g) to the high-linoleic acid-diet (7.3%) of adult male chickens counteracted the adverse effects of the linoleic acid (74).

VI. Consumer Exposure Information

In a 1965 study by Bunnell et al. the average daily consumption of alpha-tocopherol by citizens of the USA was determined to be 7.4 mg. The alpha-tocopherol content of a wide variety of foods is shown in Table 1 (340).

The following tables were compiled from data submitted by user firms. Food consumption values for each food category were derived from the Market Research Corporation of America (MRCA) data on frequency of eating and from the USDA data on mean portion size of foods in each food category. The food consumption values thus derived were coupled with the usage level data obtained in the surveys to calculate the daily intake of each substance.

Table 2 reports the usage of the tocopherols and Table 3 their use in infant formulas or baby foods. Table 11 reports the annual poundage data for the various tocopherols. Table 13 reports the possible daily intake per food category and total dietary based on food consumption by total sample.

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TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
DL-ALPHA TOCOPHEROL NAS 0226	23	BEV TYPE I(R)	*	.00000	.00000
TOCOPHEROLS CONC MIXED NAS 0227	05	MILK PRODS(R)	*	.00001	.00002
	23	BEV TYPE I(R)	*	.00001	.00004
	48	SEAS FLAVRS(R)	*	.05000	.06000
D-ALPHA TOCOPH ACET	05	MILK PRODS(R)	*	.00135	.00284
D-ALPHA TOCOPH ACET NAS 0228	11	POULTRY(R)	*	.00500	.00500
	20	GELATIN PUD(R)	*	.00100	.00100
	21	SOUPS(R)	*	.00120	.00180
	22	SNACK FOODS(R)	*	.00000	.00000
DL-ALPHA TOCOPH ACET NAS 0229	02	BREAK CERLS(R)	*	.01270	.01270
	05	MILK PRODS(R)	4	.00100	.00135
	20	IMIT DAIRY(R)	*	.01300	.01300
	48	SEAS FLAVRS(R)	*	.05000	.06000
D-ALPHA TOCOPH ACID SUCC NAS 0231	01	BAKED GOODS(R)	*	.04000	.04000

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TABLE 3 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP II) USED IN INFANT FORMULA PRODUCTS & BABY FOODS(B)

NAS SURVEY NO.	SUBSTANCE NAME	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
0227	TOCOPHEROLS CONC MIXED	83	FORMULAS(B)	*	.00122	.00146
0228	D-ALPHA TOCOPH ACET	83	FORMULAS(B)	5	.00146	.07085

TABLE 11, PART A — ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
DL-ALPHA TOCOPHEROL NAS 0226	*/ *	0	94	94			94
TOCOPHEROLS CONC MIXED NAS 0227	*/ *	800	374-	374			374
D-ALPHA TOCOPH ACET NAS 0228	7/ 8	1,250	10,979	10,981			10,981
DL-ALPHA TOCOPH ACET NAS 0229	7/ 8	450	45,734	48,134			48,134
D-ALPHA TOCOPH ACID SUCC NAS 0231	*/ *	33	33	33			33

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FCCD CATEGORY AND TOTAL DIETARY, BASED ON FCCD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FCCD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
D-ALPHA TOCOPH ACET NAS C228	20 GELATIN PUD(R)	*	0-5 MO.	.020000	.027000	.020000
			6-11 MO.	.128000	.389000	.128000
			12-23 MO.	.138000	.336000	.138000
			2-65+ YR.	.204000	.525000	.204000
D-ALPHA TOCOPH ACET NAS C228	21 SOUPS(R)	*	0-5 MO.	.002400	.010000	.003600
			6-11 MO.	.279600	.872400	.419400
			12-23 MO.	.417600	1.153200	.626400
			2-65+ YR.	.380400	1.014000	.570600
D-ALPHA TOCOPH ACET NAS C228	22 SNACK FOODS(R)	*	0-5 MO.	**********	.000000	**********
			6-11 MO.	.000000	.000000	.000000
			12-23 MO.	.000000	.000000	.000000
			2-65+ YR.	.000000	.000000	.000000
D-ALPHA TOCOPH ACET NAS C228	83 FORMULAS(B)	5	0-5 MO.	4.901220	8.979000	237.843450
			6-11 MO.	.998640	4.758140	48.461400
			12-23 MO.	.321200	.850920	15.587000
D-ALPHA TOCOPH ACET NAS C228	ALL CATEGORIES ********** ********** **********	9	0-5 MO.	5.021520	9.193000	238.045410
			6-11 MO.	2.443640	10.729990	50.975960
			12-23 MO.	1.942550	4.854120	18.229200
			2-65+ YR.	1.762650	4.807100	2.541400
DL-ALPHA TOCOPH ACET NAS C229	02 BREAK CERLS(R)	*	0-5 MO.	.076200	.215900	.076200
			6-11 MO.	2.832100	7.594600	2.832100
			12-23 MO.	3.314700	6.464300	3.314700
			2-65+ YR.	2.540000	6.570600	2.540000
DL-ALPHA TOCOPH ACET NAS C229	05 MILK PRODS(R)	4	0-5 MO.	.054000	.040000	.072900
			6-11 MO.	.624000	3.001000	.842400
			12-23 MO.	.545000	1.744000	.735750
			2-65+ YR.	.395000	1.206000	.533250
DL-ALPHA TOCOPH ACET NAS C229	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.182000	.259000	.182000
			12-23 MO.	.104000	.442000	.104000
			2-65+ YR.	.117000	.195000	.117000
DL-ALPHA TOCOPH ACET NAS C229	48 SEAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	.005000	**********
			12-23 MO.	**********	.010000	**********
			2-65+ YR.	.005000	.025000	.006000

TABLE 13, PART A — POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
DL-ALPHA TOCOPHEROL NAS 0226	23 BEV TYPE I(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.000000	.000000	.000000
			12-23 MO.	.000000	.000000	.000000
			2-65+ YR.	.000000	.000000	.000000
DL-ALPHA TOCOPHEROL NAS 0226	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.000000	.000000	.000000
			12-23 MO.	.000000	.000000	.000000
			2-65+ YR.	.000000	.000000	.000000
TOCOPHEROLS CONC MIXED NAS 0227	05 MILK PRODS(D)	*	0-5 MO.	.000540	.000400	.001080
			6-11 MO.	.000240	.000010	.012480
			12-23 MO.	.005450	.017440	.010900
			2-65+ YR.	.003950	.012000	.007900
TOCOPHEROLS CONC MIXED NAS 0227	23 BEV TYPE I(R)	*	0-5 MO.	.000240	.000360	.000960
			6-11 MO.	.002270	.007770	.010080
			12-23 MO.	.005420	.016250	.021680
			2-65+ YR.	.010400	.027770	.041600
TOCOPHEROLS CONC MIXED NAS 0227	48 SEAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	.005000	**********
			12-23 MO.	**********	.010000	**********
			2-65+ YR.	.005000	.025000	.020000
TOCOPHEROLS CONC MIXED NAS 0227	63 FORMULAS(R)	*	0-5 MO.	4.095540	7.503000	4.901220
			6-11 MO.	.234480	3.975980	.956640
			12-23 MO.	.258400	.675840	.321200
TOCOPHEROLS CONC MIXED NAS 0227	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	4.096320	7.503760	4.903260
			6-11 MO.	.842590	4.018760	1.020200
			12-23 MO.	.275270	.119330	.353780
			2-65+ YR.	.019260	.064830	.055500
D-ALPHA TOCOPH ACET NAS 0228	05 MILK PRODS(R)	*	0-5 MO.	.072500	.054000	.153360
			6-11 MO.	.842400	4.051350	1.772160
			12-23 MO.	.735750	2.354400	1.547800
			2-65+ YR.	.533250	1.628100	1.121800
D-ALPHA TOCOPH ACET NAS 0228	11 POULTRY(R)	*	0-5 MO.	.025000	.115000	.025000
			6-11 MO.	.195000	.660000	.195000
			12-23 MO.	.330000	.920000	.330000
			2-65+ YR.	.645000	1.640000	.645000

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH 1	HIGH 2
DL-ALPHA TOCOPH ACET NAS 0229	ALL CATEGORIES	7	0-5 MO.	.130200	.255900	.149100
	**********		6-11 MO.	3.631100	10.899600	3.856900
	**********		12-23 MO.	3.963700	8.660300	4.154450
	**********		2-65+ YR.	3.057000	8.004600	3.196250
D-ALPHA TOCOPH ACID SUCC NAS 0231	01 BAKED GOODS(R)	*	0-5 MO.	1.360000	1.800000	1.360000
			6-11 MO.	10.180000	20.720000	10.180000
			12-23 MO.	21.800000	35.920000	21.800000
			2-65+ YR.	54.880000	81.520000	54.880000
D-ALPHA TOCOPH ACID SUCC NAS 0231	ALL CATEGORIES	*	0-5 MO.	1.360000	1.800000	1.360000
	**********		6-11 MO.	10.180000	20.720000	10.180000
	**********		12-23 MO.	21.800000	35.920000	21.800000
	**********		2-65+ YR.	54.880000	81.520000	54.880000

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Metabolism of Alpha-Tocopherol and the Isolation of a Nontocopherol-Reducing Substance from Animal Tissues

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THE DESCRIPTION AND interpretation of the function of vitamin E in terms of a precise and well-defined biochemical reaction common to all tissues and cells still represents an unsolved problem in nutritional biochemistry.

Because of the susceptibility of vitamin E to oxidation and because of its replaceability by certain synthetic antioxidants, tocopherols are at present generally considered to be rather nonspecific physiologic antioxidants which protect the structural and metabolic integrity of intracellular units.[1–6]

Despite the attractiveness of the antioxidant theory, and even after the negative results reported on the proposed role of α-tocopherol as a cofactor of DNPH-cytochrome C-reductase,[7–10] it may still be possible to consider the participation of α-tocopherol or its active form as a cofactor in some specific reaction.[11–12]

One of the primary tasks in understanding the role of α-tocopherol either as a nonspecific antioxidant or as a cofactor would be the isolation, separation and identification of the metabolites of α-tocopherol. So far, nothing has been definitely known about the chemical transformations of α-tocopherol in the body. For this reason we have started an investigation on the metabolism of C^{14}-labeled α-tocopherol with the hope that once all metabolites have been identified one may be able to understand better the role and function of this vitamin.

It was recognized by Karrer et al.[13] and by John et al.[14] that α-tocopherol is readily oxidized to α-tocopherylquinone and that the latter can be reduced to the α-tocopherylhydroquinone in a neutral solution or reduced and recyclized to α-tocopherol in a strongly acidic solution. Boyer,[15] by the oxidation of α-tocopherol with ferric chloride in the presence of 2,2′-bipyridine in ethanolic solution, prepared a new oxidation product α-tocopheroxide which could readily be reduced to α-tocopherol or converted in an acid solution irreversibly to α-tocopherylquinone. At first Boyer believed that α-tocopheroxide, to which he had assigned an epoxy structure, represented an intermediate in the oxidation of α-tocopherol to α-tocopherylquinone. However, Martius and Eilingsfeld[16] have shown that α-tocopheroxide is a hemicetal of α-tocopherylquinone, formed only by oxidation of α-tocopherol in ethanolic solution. The possible chemical interconversions between α-tocopherol, α-tocopheroxide, α-tocopherylquinone and α-tocopherylhydroquinone are presented in classic scheme by Harrison et al.[17] (Fig. 1).

Polarographic studies by Smith et al.[18–19] and by Wachs[20] have shown that an electromotively-active bivalent reversible oxidation product is formed when α-tocopherol is oxidized at the dropping mercury electrode. Potentiometric titration[17] of α-tocopherol by auric chloride indicated an apparent oxidation-reduction potential of +0.187 volts, and the

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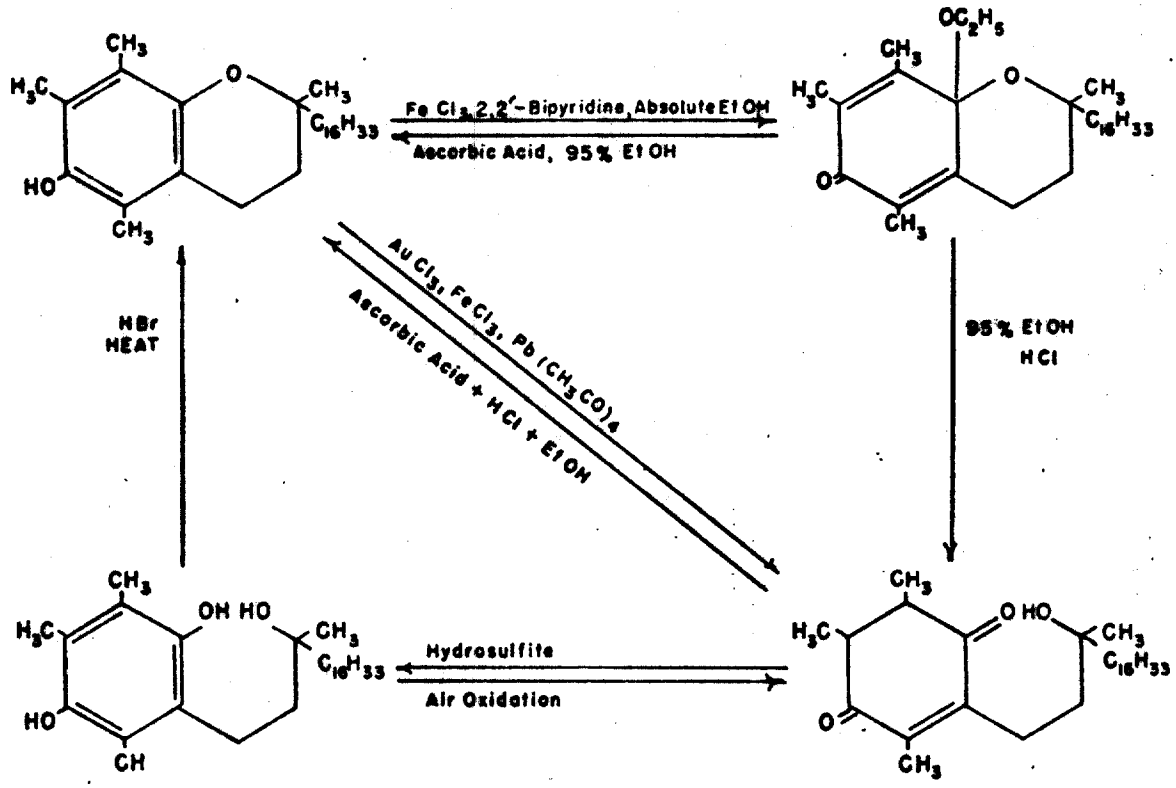

FIG. 1. The possible chemical interconversions between α-tocopherol, α-tocopheroxide; α-tocopherylquinone and α-tocopherylhydroquinone. (From: HARRISON, W. H., GANDER, J. E., BLAKELY, E. R. and BOYER, P. D. *Biochim. et biophys. acta*, 21: 150, 1956.[11])

only oxidation product formed during the titration was α-tocopherylquinone. Unfortunately, various mixtures of α-tocopherol and α-tocopherylquinone did not give any measurable potentials under the condition used for the titration, and therefore it was concluded that α-tocopherylquinone does not form an electromotively-active oxidation-reduction pair with α-tocopherol. A common intermediate, formed during the oxidation of α-tocopherol, has been proposed to account for all the chemical interconversions of known α-tocopherol compounds *in vitro*.[11,19,21] The bivalent oxidation of α-tocopherol results in the formation of a carbonium ion or tocopheryl cation (Fig. 2) which under nucleophilic attack by an ethoxy group or hydroxy group forms α-tocopheroxide or α-tocopherylquinone. This carbonium ion also represents the necessary intermediate for the reduction to α-tocopherol by ascorbic acid, stannous chloride or cysteine.

It has been generally thought that oxidation to α-tocopherylquinone represents the major step in the catabolic pathway of α-tocopherol and that further conversion to α-tocopherylhydroquinone also occurs.[1] Scudi and Buhs[22] and McCormick et al.[23] have claimed the presence of α-tocopherylquinone in human and animal plasma, Rosenkrantz et al.[24] have identified it in human stools on the basis of its

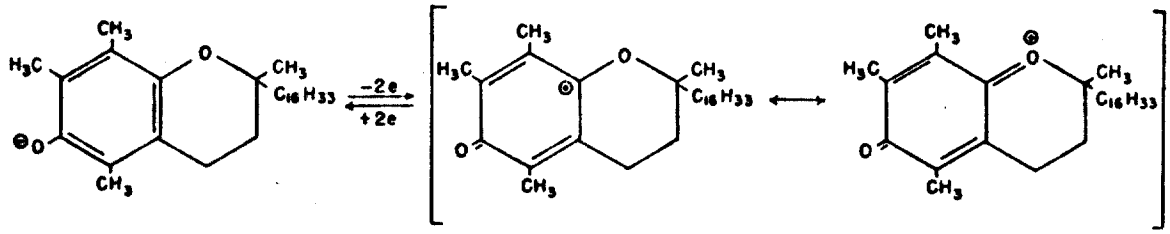

FIG. 2. The formation of α-tocopheroxide or α-tocopherylquinone from the bivalent oxidation of α-tocopherol.

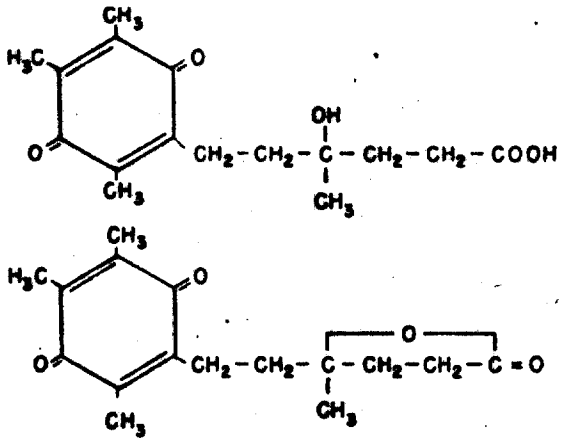

FIG. 3. Tocopherol metabolites. (From: SIMON, E. J., EISENGART, A., SUNDHEIM, L. and MILHORAT, A. T. *J. Biol. Chem.*, 221: 807, 1956.[30])

distribution constant and absorption spectra, and recently Diplock et al.[25] have reported spectroscopic and paper chromatographic evidence for its presence in horse heart mitochondria. It is interesting that Mervyn and Morton[26] found α-tocopherylquinone only in nephritic kidneys lacking α-tocopherol. On the other hand, Hines and Mattill,[27] Pollard and Bieri,[28] Slater,[11] and Draper and Alaupović[29] have been unable to demonstrate its presence in various animal tissues.

Simon et al.[30] isolated from rabbit and human urine and feces two α-tocopherol metabolites which were characterized as 2(3-hydroxy-3-methyl-5-carboxypentyl) - 3,5,6 - trimethylbenzoquinone and its γ-lactone (Fig. 3). These two compounds are the only well characterized α-tocopherol metabolites thus far discovered, and according to Simon et al. are probably formed by oxidation of α-tocopherol to α-tocopherylquinone and its subsequent reduction and conjugation with glucuronic acid, the terminal methyl group of the side chain then being oxidized to a carboxyl group which is conjugated with coenzyme A and degraded by β-oxidation until a six carbon chain is formed. The termination of β-oxidation can be explained by a nucleophilic attack by the hydroxy group on the acyl coenzyme A to form the acidic metabolite, and the quinone are formed during the isolation procedure.

Following the administration of radioactive α-tocopherol to rabbits, Martius and Costelli[4] found that, in addition to unchanged α-tocopherol, a compound could be isolated from the liver mitochondria which in its solubility properties resembled trimethylphytylbenzoquinone (Fig. 4) rather than α-tocopherylquinone. They suggested that this compound, which is reducible by acid reduction to α-tocopherol, is the functional form of vitamin E in the cells.

Sternberg and Pascoe-Dawson[32] made preliminary studies on the distribution of radioactivity in the different tissues of the rat after oral administration of C^{14}-α-tocopherol; however, these workers did not attempt to characterize any of the radioactive compounds.

In the work reported here we have administered radioactive tocopherol or tocopheryl succinate to rats and pigs. While we have been unable to detect α-tocopherylquinone or hydroquinone, or α-tocopheroxide or trimethylphytylbenzoquinone, or Simon's metabolites in the livers or tissues of these animals, we have found three metabolites, one of which (compound F) accounts for 28 to 50 per cent of the liver radioactivity.

ANIMALS AND MATERIALS

Crystalline d-α-tocopheryl-5-methyl-C^{14} succinate (obtained from Distillation Products Industries; specific activity 1.1 μc./mg.) was used for the preparation of free alcohol d-α-tocopherol-5-methyl-C^{14}, which was prepared either by saponification of the succinate ester with methanolic potassium hydroxide in the presence of pyrogallol or by reduction by lithium aluminum hydride. The free α-tocopherol was purified by chromatography on a silicic acid-Celite (2:1) column.

Normal male and female albino rats were

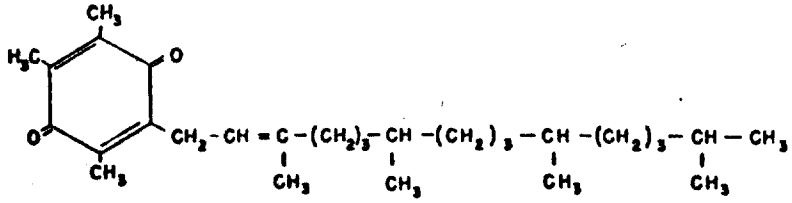

FIG. 4. Trimethylphytylbenzoquinone.

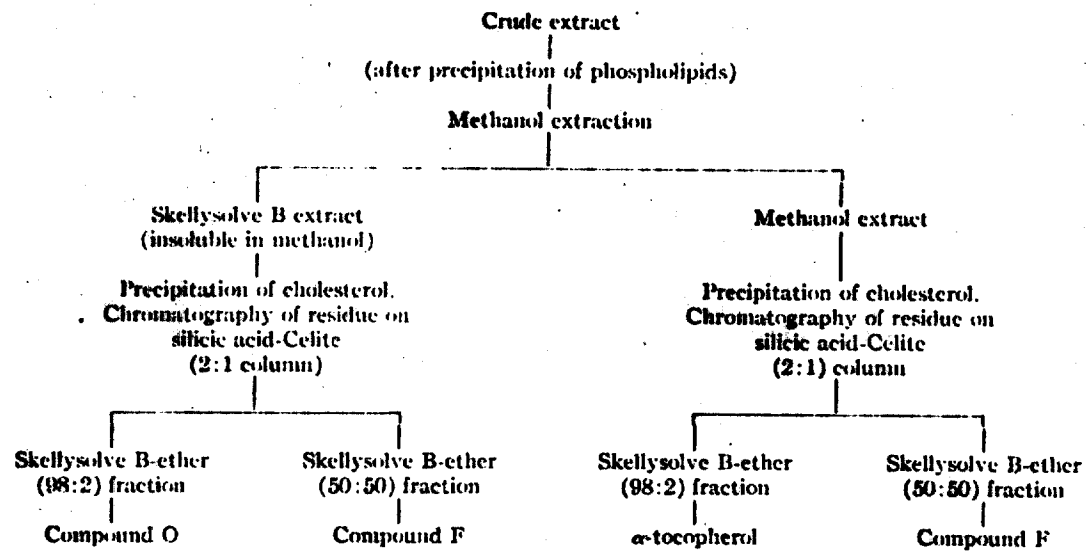

FIG. 5. Flowsheet for the isolation of α-tocopherol metabolites.

injected intraperitoneally with 1 to 2 mg. of d-α-tocopherol-5-methyl-C^{14} dissolved in 1 ml. of a mixture of 1 part of ethanol and 9 parts of 16 per cent aqueous Tween 80 solution. After forty-eight hours the animals were killed, the livers removed and immediately stored under ethanol, and the carcasses and excreta frozen.

In two experiments, normal pigs averaging 175 pounds were injected intraperitoneally with 17 to 20 mg. of labeled or unlabeled d-α-tocopherol in the same way as described for the rats. The animals were killed after forty-eight hours and the carcasses immediately frozen.

ISOLATION AND SEPARATION OF METABOLITES FROM LIVER

Individual pig livers and pooled rat livers were ground and extracted in the dark, in the presence of pyrogallol, with a mixture of 10 parts ethanol and 8.5 parts Skellysolve B for six hours in a mechanical shaker at room temperature. The extracted tissue was repeatedly washed with the same solvent and then with diethyl ether until the last traces of radioactive substances had been removed. The combined extracts were diluted with water, the Skellysolve layer was removed, and the aqueous ethanolic solution was re-extracted with Skellysolve B. The Skellysolve fractions were combined, the solvent was removed *in vacuo* under nitrogen, and the residue was dissolved in diethyl ether. All subsequent evaporations were also carried out *in vacuo* under nitrogen. The phospholipids were precipitated with acetone. The supernatant was evaporated to dryness and the residue was extracted with methanol. The methanol-insoluble residue was dissolved in Skellysolve B. The methanol and Skellysolve B extracts were chromatographed on silicic acid-Celite (2:1) columns which were developed with solvent mixtures consisting of Skellysolve B and increasing percentages of diethyl ether. The separation of unchanged α-tocopherol, and two metabolites, compound O and compound F, is shown in Figure 5.

Fractions containing compound O were purified by chromatography on silicic acid columns, and then by preparative paper chromatography, using the Eggitt and Ward[33] reverse-phase system; this was followed by chromatography on a secondary magnesium phosphate column[34,35] and precipitation in absolute ethanol at −70°c. (Fig. 6). Compound O was insoluble in ethanol at −70°c. (compound Oi) and showed a UV λ max at 283 mμ. (ethanol). Compound Os, which was soluble in ethanol at −70°c., showed a λ max at 257 mμ. ($E^{1\%}_{1cm}$ = 12.3 in isooctane).

Compound F was purified by chromatography on an alumina column and on a secondary magnesium phosphate column (Fig. 7). This oily substance showed an inflexion at 270 mμ. ($E^{1\%}_{1cm}$ = 20.1 in isooctane).

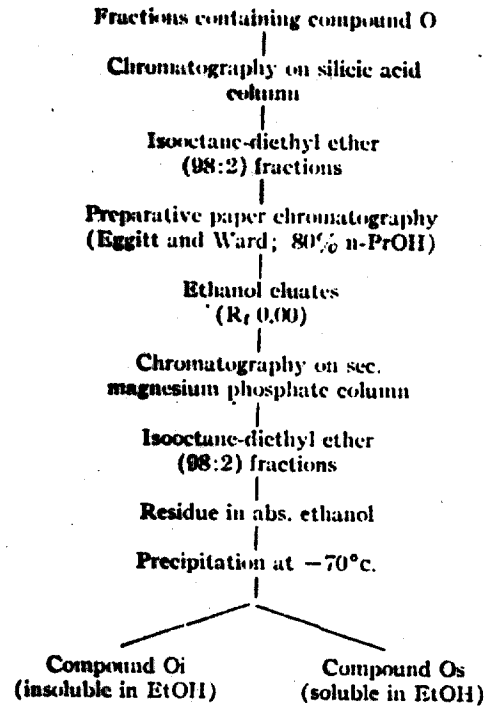

FIG. 6. Purification of compounds O.

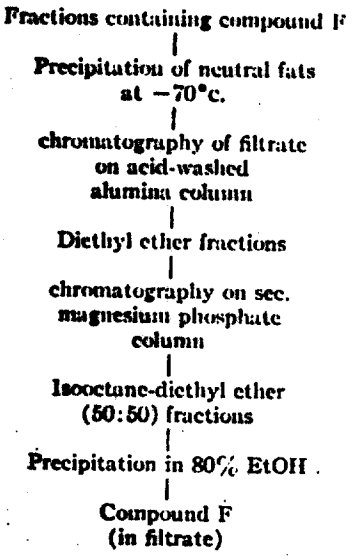

FIG. 7. Purification of compound F.

RESULTS

The amount of pure new metabolites isolated from pig and rat livers are at the present time too small for a complete chemical characterization. However, R_f values in four different paper chromatographic systems, infrared and ultraviolet spectra, and reduction in neutral and acidic solutions have been used to obtain preliminary information which enables us to compare these metabolites with the structures of known compounds related to α-tocopherol. The metabolites of vitamin E were located on the paper chromatograms by scanning for radioactivity. In Table I are presented the R_f values of the radioactive metabolites, α-tocopheryl-quinone, trimethylphytylbenzoquinone, α-tocopherol, and the neutral form of the urinary vitamin E metabolite as determined by the three reverse-phase paper chromatographic systems of Brown,[36] Eggitt and Ward[33] and Lester and Ramasarma,[37] and by the absorption paper chromatographic system of Green et al.[35] Compound Oi and compound F are not Em-

TABLE I

R_f-Values of Labeled Compounds Found in Liver Tissues as Metabolites of C^{14}-Labeled α-Tocopherol and of Some Other Related Compounds

Compound	Brown[36]		Eggitt and Ward[33]			Green[35]	Lester and Ramasarma[37]	
	90/10	50/50	75% EtOH	80% PrOH	95% PrOH		80% PrOH	95% PrOH
α-Tocopherol	0.62	0.00	0.25	0.85	...	0.75	0.83	...
Compound O (insoluble in EtOH)	0.00	0.00	0.00	0.00	0.00	0.85	0.00	0.00
Compound O (soluble in EtOH)	0.00	0.00	0.00	...	0.10	0.85	0.00	0.21
Compound F	0.89	0.30-0.45	0.65	0.88	...	0.36	0.95	...
α-Tocopherylquinone	0.85	0.00	0.50	0.93	...	0.60	0.89	...
α-Tocopherylsuccinate	0.89	...	0.77	...	...	0.00	0.95	...
Trimethylphytylbenzo-quinone	0.22	0.00	0.02	0.40	...	0.83	0.46	...
Urinary vitamin E metabolite (neutral form)	S.F.	S.F.	0.88	...	...	...	...	...

TABLE II
Distribution of Radioactivity Expressed as Per Cent

Species	Compound F	Compound O	α-Tocopherol
Pig liver	50.4	1.2	48.4
Rat liver	21.0	5.5	73.5

merie-Engel-positive and differ from each other in their elution patterns on silicic acid-Celite columns, in their ultraviolet adsorption spectra, and in their R_f values in all paper chromatographic systems. Compound Oi and Os (also Emmerie-Engel negative) differ in their ultraviolet absorption spectra, in their solubility properties at low temperatures, and in their R_f values in the reverse phase chromatographic system of Lester and Ramasarma developed with a mixture of 95 per cent n-propanol and 5 per cent water. The R_f values for compounds O and F differ from those of the urinary metabolite of vitamin E, trimethylphytylbenzoquinone, α-tocopheryl succinate and α-tocopherylquinone. Compound F represents the major metabolite isolated from both rat and pig liver, although its absolute amount is extremely small. It is possible to obtain 350 to 400 μg. of crude compound F from a pig liver and 8 to 12 μg. from a rat liver. The conversion of α-tocopherol to compound F and to compounds O was calculated from the percentage of radioactivity recovered for each compound after separation on the columns and indicates considerable conversion of α-tocopherol into compound F (Table II). The O compounds are clearly minor metabolites in both species.

Having found that compound F is a major α-tocopherol metabolite in pig and rat liver, it was important to prove whether or not it is identical with α-tocopherylquinone or trimethylphytylbenzoquinone, which have been considered as major catabolic products[1] or have been suggested as possible functional forms of α-tocopherol.[31] Mixtures of these three compounds were chromatographed on Brown paper and developed with a mixture of 90 per cent acetonitrile and 10 per cent water, and a mixture of 50 per cent acetonitrile and 50 per cent water. In the former case a clear separation was achieved between trimethylphytylbenzoquinone (R_f 0.22) and

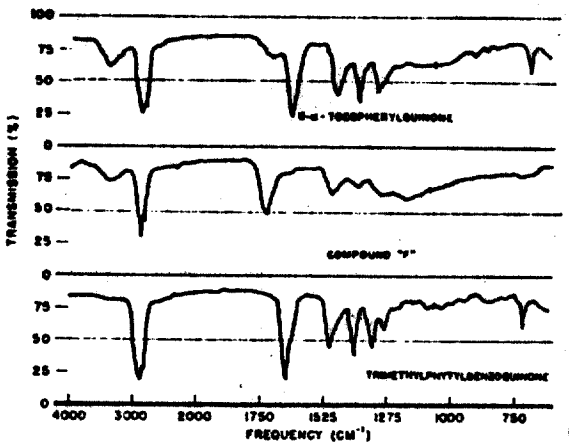

FIG. 8. Infrared spectra of d-α-tocopherylquinone, compound F and trimethylphytylbenzoquinone.

α-tocopherylquinone (R_f 0.85) and compound F (R_f 0.89), in the latter case compound F was separated (R_f 0.3 −0.45) from a mixture of the two quinones (R_f 0.0 and 0.0). In the Eggitt and Ward system, developed with 75 per cent ethanol, a mixture of compound F and α-tocopherolquinone always gave two close but distinct spots (R_f 0.50 and 0.65).

Additional support for a structural difference between compound F and these quinones may be drawn from the infrared spectra (Fig. 8). The typical α,β-unsaturated carbonyl band at 1644 cm.$^{-1}$ present in both α-tocopherylquinone and trimethylphytylbenzoquinone is lacking in compound F. The infrared spectrum of compound F indicated the presence of a hydroxyl group (3400 cm.$^{-1}$), and a very strong band at 1740 cm.$^{-1}$ due to the carbonyl group. There is a slight indication of aromaticity (band at 1600 cm.$^{-1}$, C−C vibration) and strong evidence for the presence of methylene groups (C–H vibrations at 2910 cm.$^{-1}$ and 725 cm.$^{-1}$).

The infrared spectrum of compound Oi is compared in Figure 9 with the infrared spectra of α-tocopherol and α-tocopheryl acetate. On the basis of this spectra it appears that compound Oi contains no hydroxyl group but does contain a carbonyl group.

Compound F and compound Oi were treated with stannous chloride in strongly acidic solution, and with lithium aluminum hydride in neutral solution. The reaction products were isolated and characterized by their R_f values on the Eggitt and Ward system. While compound Oi was unchanged, compound F yielded

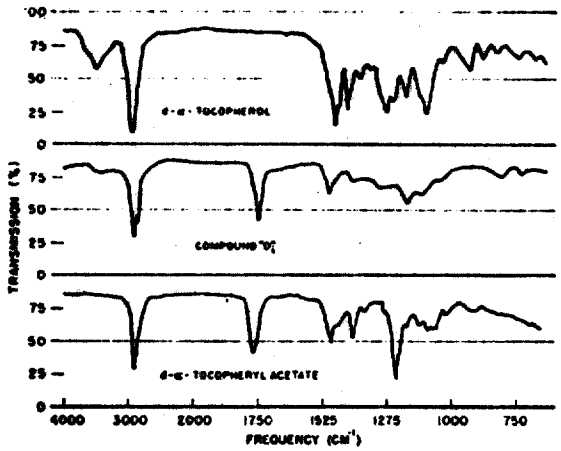

FIG. 9. Infrared spectra of d-α-tocopherol, compound Oi and d-α-tocopheryl acetate.

in both reduction procedures Emmerie-Engel-positive compounds, neither of which was identical with α-tocopherol. In Table III are presented the R_f values of the reduction products of compound F, α-tocopherylquinone and trimethylphytylbenzoquinone. The two latter quinones form α-tocopherol in acidic solution and their respective hydroquinones in neutral solutions.

Neither compound F nor the O compounds show the presence of phosphorus in their molecules as determined by the method of Hanes and Isherwood.[39]

COMMENTS

In the isolation procedure which has been briefly described, all necessary precautions were taken to eliminate the possibility of the formation of artifacts. Emmerie and Engel[40] and Moss et al.[41] have shown that tocopherols are sensitive to alkali, but the losses can be diminished in the presence of antioxidants. Mervyn and Morton[26] have studied the effect of pyrogallol as an antioxidant on the analysis of kidney tissue with and without added tocopherol. When the tissue was digested with premixed 60 per cent ethanolic KOH and pyrogallol they were able to recovery 88 per cent of added α-tocopherol. In our experiments on the saponification of labeled α-tocopheryl succinate with the same premixed solution and subsequent purification of the reaction mixture on the silicic acid-Celite column only 40 to 50 per cent of the α-tocopherol was recovered. In contrast, about 95 per cent recovery

TABLE III

R_f-Values of the Reduction Products of Compound F and Quinones in Eggitt and Ward Paper[33]

Compounds	Reduction with $SnCl_2$ + HCl		Reduction with $LiAlH_4$ (75% EtOH)
	75% EtOH	80% n-propanol	
Compound F	0.00*	0.89*	0.89*
α-Tocopheryl-quinone	0.25†	0.85†	0.00‡
Trimethylphytyl-benzoquinone	0.25†	0.85†	0.00*

* Emmerie-Engel-positive reaction.
† α-Tocopherol.
‡ α-Tocopherylhydroquinone.

of α-tocopherol was achieved by reduction of the succinate ester with lithium aluminum hydride followed by silicic acid-Celite column chromatography. In saponification experiments several artifacts were eluted from the silicic acid-Celite column. One of these was identified by R_f values, ultraviolet absorption spectra and reducibility in acidic solution to α-tocopherol as α-tocopherylquinone. Another compound showed an R_f of 0.0 in the Eggitt and Ward system and 0.8 in the system of Lester and Ramasarma (95 per cent n-propanol as developing solvent), and thus could be distinguished from compound O.

In the experiments in which α-tocopheryl succinate was reduced by lithium aluminum hydride and the products were separated by chromatography on a silicic acid-Celite column, only small amounts of these artifacts were obtained. These artifacts were distinguished from the true metabolites by ultraviolet absorption and R_f values. In all our experiments the saponification of tissue or extracts was avoided not only because of the possible degradation of α-tocopherol but also because of the sensitivity of the metabolites to alkali. It was necessary to remove phospholipids by precipitation and to eliminate, stepwise, neutral fats, free and esterified cholesterol, and other fat-soluble substances by using different columns and paper chromatographic systems, since these would contaminate the fractions containing the α-tocopherol metabolite. In contrast to the recent report

by Diplock et al.,[29] but confirming previous findings[11,27-29] we were unable to find any detectable α-tocopherylquinone in rat or pig livers. It is possible that α-tocopherylquinone might be formed in the intestine[24] or in tissues under pathologic conditions,[26] although Mervyn and Morton did not find it in all nephritic kidneys. Uncontrolled tissue saponification leads to the partial destruction of α-tocopherol and to the formation of α-tocopherylquinone and other unidentified artifacts. Our studies have shown that whenever one has to rely upon paper chromatographic methods for identification of extremely small amounts of structurally very similar compounds, several systems must be used. It has been found that by the oxidation of ubichromenol or ubichromanol compounds analogous to α-tocopherylquinone are formed, which show in some reverse-phase chromatographic systems the same R_f values as α-tocopherylquinone.[42]

It has been shown that neither Simon's metabolites nor trimethylphytylbenzoquinone are present in liver tissue and thus cannot be considered as functional forms of vitamin E. The major metabolite, compound F, is neither a quinone nor a hydroquinone, and contains a long saturated carbon chain, a carbonyl group, and possibly a benzene ring in its molecular structure. It can be readily reduced to two different Emmerie-Engel-positive compounds, depending on the reduction method, neither compound being α-tocopherol.

Preliminary studies on the distribution of the α-tocopherol metabolites in subcellular fractions[29] have indicated the presence of compound F and α-tocopherol in liver mitochondria. Although it has been shown that α-tocopherol does not form a redox pair with compound F and does not participate as a carrier in an electron transport system, one may speculate that possibly compound F and its reduction product function as a redox pair either in an electron transport system or in some other biological system. In such a case one might explain the role of α-tocopherol in acting as a physiologic antioxidant and as the starting material for the formation of compound F, which then participates with its reduction product in a specific reaction. Absence of phosphorus in compounds F and O does not support the suggestions for a possible participation of α-tocopherol or its metabolites in oxidative phosphorylation.[11] On the basis of our results it is somewhat difficult to visualize the role of α-tocopherol as only that of an unspecific antioxidant because of the absence of such typical oxidation products as α-tocopheryl-quinone or tocored or tocopurple[43] in animal tissues. These should have been formed had α-tocopherol only been protecting the cellular units from the accumulation of lipid peroxides. Inglett and Mattill[44] and Harrison et al.[17] have demonstrated that the oxidation of α-tocopherol by benzoyl peroxide, cytochrome C or hydrogen peroxide results primarily in the formation of α-tocopherylquinone.

It is hoped that further characterizations of the metabolites (compound F and O) following their isolation in sufficient quantities will throw more light on the *in vivo* function of vitamin E.

THE ISOLATION OF A NEW REDUCING COMPOUND FROM ANIMAL TISSUES

It has been shown that the synthetic antioxidants *N,N'*-diphenyl-p-phenylene-diamine (DPPD)[46] and methylene blue (MB)[45] can prevent and cure the reproductive failure of vitamin E deficiency in female rats.

In further extension of these studies it has been found that rats carried through resorption-gestation on a vitamin E-deficient diet will respond in subsequent reproductive cycles to the antioxidants DPPD and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (Ethoxyquin)[47] bearing live young. The antioxidants prevented the resorption-gestation symptoms of vitamin E deficiency when fed from the beginning of the experiment to rats in E-free rations, and this protective effect was carried through at least two generations when DPPD was continuously included in the diet at levels similar to those required for vitamin E.[46,47,48] Since rats could be maintained and carried through two generations on a vitamin E free diet supplemented with synthetic antioxidants, it became important to determine whether the antioxidant was itself responsible for the cure, or whether it was merely functioning to preserve minimal amounts of vitamin E or its metabolites which might be left in the body tissues.

TABLE IV
R_f Values of Emmerie-Engel-Reactive Compound Present in Liver Extracts

Diet	Treatment	Eggitt and Ward[33]		Silicone[37] n-PrOH (80%)
		EtOH (75%)	n-PrOH (80%)	
Vitamin E free	None	0.00*	0.53*	0.77
Vitamin E free	α-Tocopherol	0.00*	0.53*	0.78
		0.25	0.85	0.84
Vitamin E free	DPPD	0.00*	0.53*	0.77
		0.83	0.87	0.84
Vitamin E free	BHT	0.00*	0.53*	0.77
		0.95	0.95	...
Vitamin E free	Ethoxyquin	0.00*	0.53*	...
		0.84	0.90	...
Checkers	None	0.00*	0.53*	0.74

* These are initial values. Final values after repeated chromatography and purification are 0.79 and 0.90.

Female rats maintained on the vitamin E free synthetic diet[42] were killed at the end of each experiment, and the livers were removed and analyzed for vitamin E.[47,48] Homogenized livers were extracted with foaming solvent in the presence of pyrogallol. The extract was not saponified, because of the destructive action of alkali on α-tocopherol and its metabolites and also because of possible formation of ubichromenol or some other reducing artifacts which could be formed from ubiquinone in boiling alkaline ethanol solution.[49–51] The residue was dissolved in absolute ethanol, and the neutral fats were precipitated by cooling the solution in dry ice-acetone. The filtrate was purified by subsequent reverse phase paper chromatography, using the systems of Eggitt and Ward[33] and Lester and Ramasarma.[37] The R_f values of the Emmerie-Engel reactive compounds found in liver are presented in Table IV. It was found that animals on vitamin E free diets or even on a low E diet (Checkers) contained no detectable α-tocopherol in their livers and carcasses. If α-tocopherol were added to the diet it could be found and quantitatively determined in the animal tissues. Synthetic antioxidants, DPPD, Ethoxyquin (EMQ), and BHT (2,6-di-tert-butyl-4-methyl-phenol) occurred in the liver tissues and could be quantitatively determined when given to the animals. It was found that, in addition to these administered antioxidants, all rats contained an additional Emmerie-Engel-positive material (compound X), whether or not vitamin E or any antioxidant was fed. Furthermore, it was found that the level of compound X in rat liver tissue appeared to be related to the level of α-tocopherol fed.[48] The concentration of compound X in vitamin E deficient rat liver was 9.0 μg./gm. of fresh tissue calculated as vitamin E.

Hines and Mattill,[27] using the Emmerie-Engel assay method for vitamin E in rat and rabbit liver and muscle tissue, showed that the level of vitamin E so determined was related to the level of vitamin E in the diet. The concentrations of total "vitamin E" (total Emmerie-Engel-reactive material) in liver tissues of animals fed high vitamin E, normal, and vitamin E deficient diets were: for the rabbit, 86.8, 9.2 and 9.4; and for the rat, 42.3, 22.1 and 22.6 μg./gm., respectively. These authors assumed that their figures represented only tocopherols. Hickman, Kaley and Harris[52] observed the presence of a substance with reducing properties in extracts of rat and human feces which gave "apparent" tocopherol values when assayed by the Emmerie-Engel method. They found that when the subjects were given higher doses of α-tocopherol the excretion of α-tocopherol and of the other reducing substance increased proportionally to the amount of α-tocopherol administered. Without any further studies on the identification of this substance, they concluded that it was a labile substance spared by the tocopherol in the alimentary tract. Rosenkrantz, Milhorat and Farber[24] confirmed the presence of a heat-labile, alcohol-soluble reducing substance in human stools but attempts to identify this material were unsuccessful. Bolliger and Bolliger-Quaife[53] reported that chromatography of various tissue extracts gave two Emmerie-Engel-reactive compounds of which only one was α-tocopherol. Very recently, Bieri and his co-workers[54,55] have found that liver and heart tissues of chickens raised on vitamin E free diets contained Emmerie-Engel-reactive material which did not chromatograph as α-tocopherol.

Slater and his co-workers[11] have shown that the increase in apparent α-tocopherol content of tissues analyzed by the method of Bouman

TABLE V
R_f Values of Emmerie-Engel-Reactive Compounds

Compounds	Eggitt and Ward[23]		Silicone[37]	Green[38]
	EtOH (75%)	n-PrOH (80%)	n-PrOH (80%)	
α-Tocopherol	0.25	0.85	0.85	0.75
DPPD*	0.83	0.95	0.84	0.82
Ethoxyquin†	0.84	0.90	0.91	...
BHT	0.95	0.95	0.80	...
Ubichromenol-50	0.00	0.40	...	...
Ubichromanol-50	0.00	0.34	0.62	0.73
Ubiquinol-50	0.00	...	...	0.73
Compound X	0.00 (initial)	0.53 (initial)	0.80–0.90	0.78
	0.79 (final)	0.90 (final)	...	...

* N,N′-diphenyl-p-phenylenediamine.
† 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (Santoquin).

et al.,[56] which employs a preliminary treatment of the tissue extract with ascorbic acid in HCl, was due to the formation of acid-reduced ubiquinone, an Emmerie-Engel-positive cyclization product of ubiquinone. The same compound was found to be present in all extracts which were passed through the Florex XXS column.[48,57] Laidman et al.[58] have isolated and identified an Emmerie-Engel-reactive compound, present in the unsaponifiable matter of kidney and other tissues of many species as a cyclic isomeride of ubiquinone 50 (ubichromenol) which contains a chromene ring and a side chain consisting of nine isoprenoid units. Diplock et al.[59] have found that the mixture of ubiquinones 50, 45, 40 and 35 in rat tissues is accompanied by a similar mixture of ubichromenols. They observed that ubichromenol is present only if the preparation contains the corresponding ubiquinone. Ubihydroquinones (ubiquinols),[60] ubichromenols, ubichromanols, vitamin A and carotenoids are so far the only well characterized nontocopherol reducing compounds possibly present in animal tissues. The natural occurrence of ubichromenols is still unsolved,[49-51] and ubichromanols if present in extracts must be considered as artifacts. The R_f values of these reducing compounds are given in Table v.

The new unknown reducing substance,

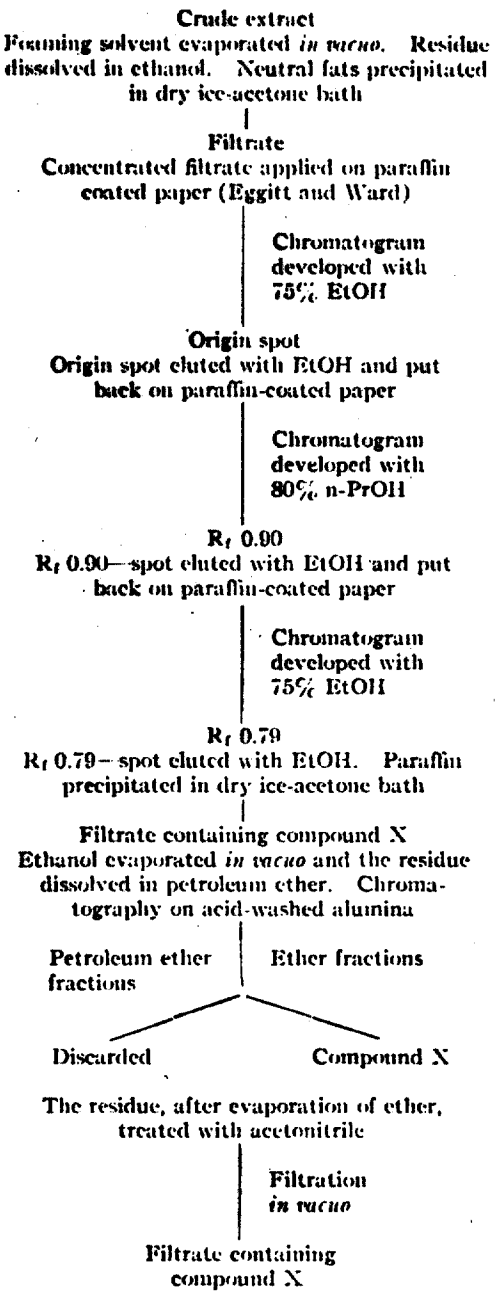

FIG. 10. Flowsheet for the isolation of compound X.

compound X, differs distinctly from ubichromenol, ubichromanol and tocopherols in reverse phase paper chromatographic systems. This compound has been found in rat, pork, beef and chicken liver and beef heart mitochondria. The isolation procedure is outlined in Figure 10. It does not include alkaline saponification, excessive heat or column chromatography on Florex XXS, avoiding thus the conditions for the possible formation of ubichromanol or other artifacts. The neutral

fats, cholesterol and cholesterol esters are separated by precipitation from ethanol solution in dry ice-acetone, and vitamin A, carotenoids and ubichromenol are then separated from compound X on paper. The final purification is achieved by chromatography on a deactivated (10 per cent) alumina column. Compound X is a pale yellow oily substance characterized by: an inflexion at 271 mμ. and R_f values of 0.79 and 0.90 in Eggitt and Ward system chromatograms (developed with 75 per cent EtOH and 80 per cent n-propanol, respectively); 0.80 on silicone-treated paper (developed with 80 per cent n-propanol); and 0.78 in the Green system. Its infrared spectrum shows the presence of a hydroxyl group (3400 cm.$^{-1}$) and a strong C=O band (1735 cm.$^{-1}$). Green et al.[51] suggested that compound X may be ubichromenol 45 or 50. The presence of a strong carbonyl band, different R_f values and a different ultraviolet absorption differentiate compound X from the ubichromenols. Very recently, Brand, Dahl and Mahler[61] reported the presence of a rapidly migrating component with an R_f value 0.90 – 0.93 on the paper system of Lester and Ramacarma, developed with 80 per cent n-propanol. This material, which was not reduced by KBH_4 but was capable of interacting with neotetrazolium chloride, may be similar to or identical with compound X. The authors considered their compound to be ubichromenol or a derivative of it, such as phosphate ester. It has been found that compound X does not contain phosphorus.

There is no precursor product relationship between compound X and α-tocopherol, since compound X is not labeled following administration of C^{14}-α-tocopherol. It may be possible that compound X is an important functional compound in the body and that vitamin E and other active antioxidants function in the reduction of this compound to this active form or in the protection of this compound, which is formed by the animal body.

SUMMARY

It has been demonstrated that α-tocopherol-5-methyl-C^{14} was converted in rats and pigs into three metabolites: compound F and compounds Oi and Os. These compounds are oils, and they were isolated and separated from liver extracts by a combination of column and paper chromatographic methods. Compound F, which is the main metabolite, can be reduced readily to an Emmerie-Engel-positive material. Neither compound is a quinone or hydroquinone, and neither contains phosphorus in its molecule. It has been shown that the compounds α-tocopherylquinone, α-tocopheryl-hydroquinone, α-tocopheroxide, trimethyl-phytylbenzoquinone and Simon's metabolites, which have been suggested as possible metabolic or degradation products of α-tocopherol, are not present in rat and pig liver under conditions used in these experiments. The importance of these compounds in studying the function of vitamin E has been discussed.

The isolation of a new reducing compound from rat, chicken, and beef liver and from beef heart mitochondria has been described and its possible relationship with α-tocopherol and other antioxidants discussed.

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Effect of Dietary Linoleic-Acid, Vitamin-E and Ethoxyquin on Fertility of Male Chickens[1]

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ABSTRACT Adult male chickens were fed diets low in linoleic acid, vitamin E and ethoxyquin or high in linoleic acid with or without added vitamin E or ethoxyquin for 25 weeks. Fertilizing capacity and sperm concentration of semen were adversely affected with males fed the high linoleic acid diet without vitamin E or ethoxyquin. The addition of vitamin E or ethoxyquin to the high linoleic acid diet overcame these adverse effects. The low linoleic acid diet without vitamin E and ethoxyquin had no adverse effect on fertilizing capacity and concentration of semen. No differences were evident for males fed any of the diets as far as semen volume, hatchability of fertile eggs, body or testes weights or feed consumption were concerned. During the 26th and 27th weeks increasing the number of sperm inseminated failed to improve fertility of the males fed the diet high in linoleic acid without vitamin E. The results indicate that diets high in linoleic acid but without vitamin E affect fertilizing capacity as well as the number of sperm produced.

Adamstone and Card (1) reported testicular degeneration resulting after a vitamin E-deficient diet was fed to Rhode Island Red males for a 2-year period. Although no differences were observed at one year, an adverse effect on fertilizing capacity was noted with a limited number of males after 2 years. More recently Machlin et al. (2) reported adverse effects on fertility as well as egg production of White Leghorn layers fed diets low in vitamin E but containing a high level of linoleic acid. They showed that lowered fertility and egg production were overcome by addition of vitamin E or ethoxyquin to the diet. In the absence of the high level of linoleic acid as well as vitamin E and ethoxyquin no adverse effects on fertility or egg production could be observed during the 6 weeks of their experiment.

The relationship of vitamin E to linoleic acid metabolism has been reviewed by Scott (3). Evidence is cited indicating that vitamin E functions as a biological antioxidant that protects against encephalomalacia in chicks by preventing the breakdown of linoleic acid to 12-oxo-*cis*-9-octadecenoic (keto) acid. Whether or not the same relationship exists with adult poultry has not been established, but the data reported with layers (2) suggest such a possibility.

In view of a previous interest in the area of nutrition as related to male fertility (4), the present experiment was undertaken to determine whether linoleic acid would exert a detrimental effect on fertilizing capacity of adult male chickens fed diets with or without vitamin E or ethoxyquin.

EXPERIMENTAL

Twenty-four dubbed White Leghorn cockerels hatched February 20, 1963, were housed in individual wire-floor cages on July 27, 1963. The present experiment commenced March 31, 1964, at which time the males were divided into 4 comparable groups and fed the experimental diets shown in table 1.[2] The males were distributed into their respective groups in such a way that differences in body weights, as well as semen volume and fertility (based on 3 preliminary ejaculations and inseminations) were minimized. In addition the males on the 4 treatments

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[2] Prior to this date the males had previously been divided into 3 comparable groups beginning November 26, 1963, during which time they were fed rations containing varying protein levels for 7 weeks. For 18 weeks prior to the commencement of the present trial all birds were fed an adequate control ration free-choice.

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TABLE 1

Composition of experimental diets

	Diet no.			
	1	2	3	4
	%	%	%	%
Isolated soybean protein [1]	25.0	25.0	25.0	25.0
Coconut oil, air-oxidized [2]	10.0	—	—	—
Safflower oil, air-oxidized [3]	—	10.0	10.0	10.0
Glucose monohydrate [4]	48.7	48.7	48.2	48.4
Cellulose [5]	8.0	8.0	8.0	8.0
Methionine hydroxy analogue [6]	0.4	0.4	0.4	0.4
Vitamin A, dry (10,000 IU/g)	0.2	0.2	0.2	0.2
Vitamin D_3, dry (15,000 ICU/g)	0.025	0.025	0.025	0.025
Vitamin mixture [7]	0.6	0.6	0.6	0.6
Vitamin E (44 IU/g) [8]	—	—	0.5	—
Choline chloride (25%)	1.0	1.0	1.0	1.0
Salts N [9]	6.0	6.0	6.0	6.0
Na_2SeO_3 in glucose monohydrate [10]	0.1	0.1	0.1	0.1
Ethoxyquin [11]	—	—	—	0.3
	100.0	100.0	100.0	100.0
Calculated analysis: [12]				
Linoleic acid, %	0.03	7.3	7.3	7.3
Vitamin E, mg/kg	0.9	4.3	166.3	4.3

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[2] Konut, Durkee Famous Foods, Glidden Company, Berkeley, California.
[3] Alkaline refined safflower oil, Pacific Vegetable Oil Corporation, San Francisco.
[4] Cerelose 2001, Corn Products Company, New York.
[5] Solka Floc BW-100, Brown Company, Berlin, New Hampshire.
[6] Ca DL-2-hydroxy,4-methylthiobutyrate; provided by Monsanto Chemical Company, St. Louis.
[7] Vitamin mixture supplied: (in mg/kg of diet) vitamin K (menadione), 1.2; vitamin B_{12}, 0.036; thiamine·HCl, 28.8; riboflavin, 19.2; Ca D-pantothenate, 24; niacin, 120; pyridoxine·HCl, 9.6; folacin, 4.8; and biotin, 0.36 (Nutritional Biochemicals Corporation, Cleveland).
[8] Myvamix; provided by Distillation Products Industries, Rochester, New York.
[9] Salts N supplied: (as % of diet) Ca, 1.24; P, 0.8; K, 0.37; Na, 0.384; Cl, 0.58; Mg, 0.08; Fe, 0.00334; Mn, 0.00813; I, 0.0008; Zn, 0.00728; and Cu, 0.0004 (Nutritional Biochemicals Corporation).
[10] Optional trace mineral, with 21.9 mg of Na_2SeO_3 mixed per 100 g glucose monohydrate.
[11] Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline); provided by Monsanto Chemical Company, St. Louis.
[12] Based on values cited for ingredients in table 2.

were placed in the 3 tiers of a battery in such a way as to avoid positional effects as much as possible. Data were collected on all groups for 25 weeks. After this period additional data were obtained for the groups receiving the high level of linoleic acid with and without supplemental vitamin E. All males were killed on October 16, 1964, at which time testes weights were obtained.

The rations used are shown in table 1 and were adapted from the report of Machlin et al. (2) as diet S-25-B modified by reducing the calcium and phosphorus levels to values slightly above those specified for chicks (5) through the use of salts N (6) [3] including an optional level of selenium (0.1 mg/kg of diet). Linoleic acid values were varied by using either coconut [4] or safflower [5] oil (diets 1 vs. 2, 3 and 4). The tocopherol content of the coconut or safflower oil was reduced by bubbling air from an aspirator pump through a 4.54-kg sample of the respective oil contained in a 12-liter round-bottom flask enclosed in an electric heating mantle for 32 hours at 72°. Following this treatment, 0.1% of an antioxidant mixture [6] was added to minimize subsequent development of rancidity (2). The oils were stored in the deep freeze (– 12°) until required. The α-tocopherol and linoleic acid content [7] of selected ingredients used in these rations is shown in table 2. The calculated vitamin E and linoleic acid content of the diets resulting from these analyses is shown in table 1. The addition of 162 mg of vita-

[3] See footnote 9, table 1.
[4] See footnote 2, table 1.
[5] See footnote 3, table 1.
[6] Tenox 6 (Eastman Chemical Products, Inc., Kingsport, Tennessee) which contains: (in per cent) butylated hydroxytoluene, 10; butylated hydroxyanisole, 10; propyl gallate, 6; citric acid, 6; propylene glycol, 12; and mixed glycerides, 56.
[7] The authors are grateful to Mr. R. Bull, Department of Animal Science and Dr. I. J. Tinsley, Department of Agricultural Chemistry for conducting the analyses for α-tocopherol and linoleic acid, respectively.

TABLE 2

α-Tocopherol and linoleic acid content of selected ingredients used in the diets

	α-Tocopherol	Linoleic acid
	μg/g	%
Coconut oil	21.5	0.3
Coconut oil, air-oxidized	7.4	0.3
Safflower oil	490.0	75.0
Safflower oil, air-oxidized	41.1	72.6
Isolated soybean protein	0.8	—
Cellulose	0	—
Glucose monohydrate	0	—

min E[8]/kg of diet (diet 3) or 0.3% of ethoxyquin[9] (diet 4) was made at the expense of glucose monohydrate.[10] The rations were mixed in 45.4-kg lots as needed and stored in the deep freeze. Estimated amounts to be consumed weekly were kept in the room with the males for use during that week.

Care and management of these males have been described previously (8). Both feed and water were provided ad libitum. Body weights and feed consumption data were obtained bi-weekly starting with the first week. Usually, groups of 3 White Leghorn layers each were artificially inseminated every 4 weeks with 0.05 ml of undiluted semen from individual males; however, in a few instances only 1 or 2 females could be inseminated when limited amounts of semen were available or when mortality had occurred among the females. The hens were rotated so that no male's semen was used for insemination of the same group of hens twice. No fertility data were obtained during the seventeenth week of the experiment because the yearling hens were replaced by young pullets which were not yet laying at an adequate rate. A total of 2,153 eggs was incubated during this experiment.

In addition to obtaining data on semen volume, fertility and hatchability of fertile eggs, data on semen concentration were also obtained turbidometrically (9). Semen samples from individual males were stored overnight in a refrigerator (5°), diluted 1:100 using 0.05 ml of semen in 0.85% saline solution and transmittancy readings obtained using tubular absorption cells in a Cenco-Sheard-Sanford photometer with a 640-mμ filter. All such readings were subsequently converted to optical density values (10) for expression on a linear basis.

After 25 weeks, the hens were inseminated on 2 separate occasions with semen (0.03 to 0.1 ml) from males receiving the high level of linoleic acid with and without vitamin E (2 hens/male). This was done to compare fertility resulting from inseminations of more comparable numbers of sperm from the 2 groups since sperm concentration of semen from the high linoleic acid — low vitamin E males was lower than that of the group with vitamin E.

The data were subjected to regression analysis with statistical significance determined by *t* or *F* tests.

RESULTS AND DISCUSSION

The results for the first 25 weeks of this experiment are shown in figure 1. A significant decrease ($P < 0.01$) in fertilizing capacity of semen was observed when males were fed diet 2, containing a high level of linoleic acid (7.3%), a low level of vitamin E (4.3 mg/kg) and no ethoxyquin (fig. 1A). A marked decrease occurred after 5 weeks and at 25 weeks all eggs were infertile as determined by macroscopic examination of the germinal disc for all clear eggs candled out. However, diet 3, containing high levels of linoleic acid and vitamin E (166.3 mg/kg) but no ethoxyquin, maintained fertilizing capacity as did diet 4, supplemented with 0.3% ethyoxquin but no vitamin E. Diet 1, containing low levels of linoleic acid (0.03%) and vitamin E (0.9 mg/kg) as well as no ethoxyquin, also maintained fertilizing capacity throughout the experiment.

Semen volume appeared unaffected throughout the test period for all treatments used (fig. 1B). On the other hand, semen density decreased significantly ($P < 0.01$) for the males fed a high level of linoleic acid without supplemental vitamin E or ethoxyquin (fig. 1C). In this group a marked decrease was noted after 13 weeks. A significant difference ($P < 0.05$) in hatchability of fertile eggs (fig. 1D) was noted with males fed at the high level of linoleic acid without supplemental vita-

[8] See footnote 8, table 1.
[9] See footnote 11, table 1.
[10] See footnote 4, table 1.

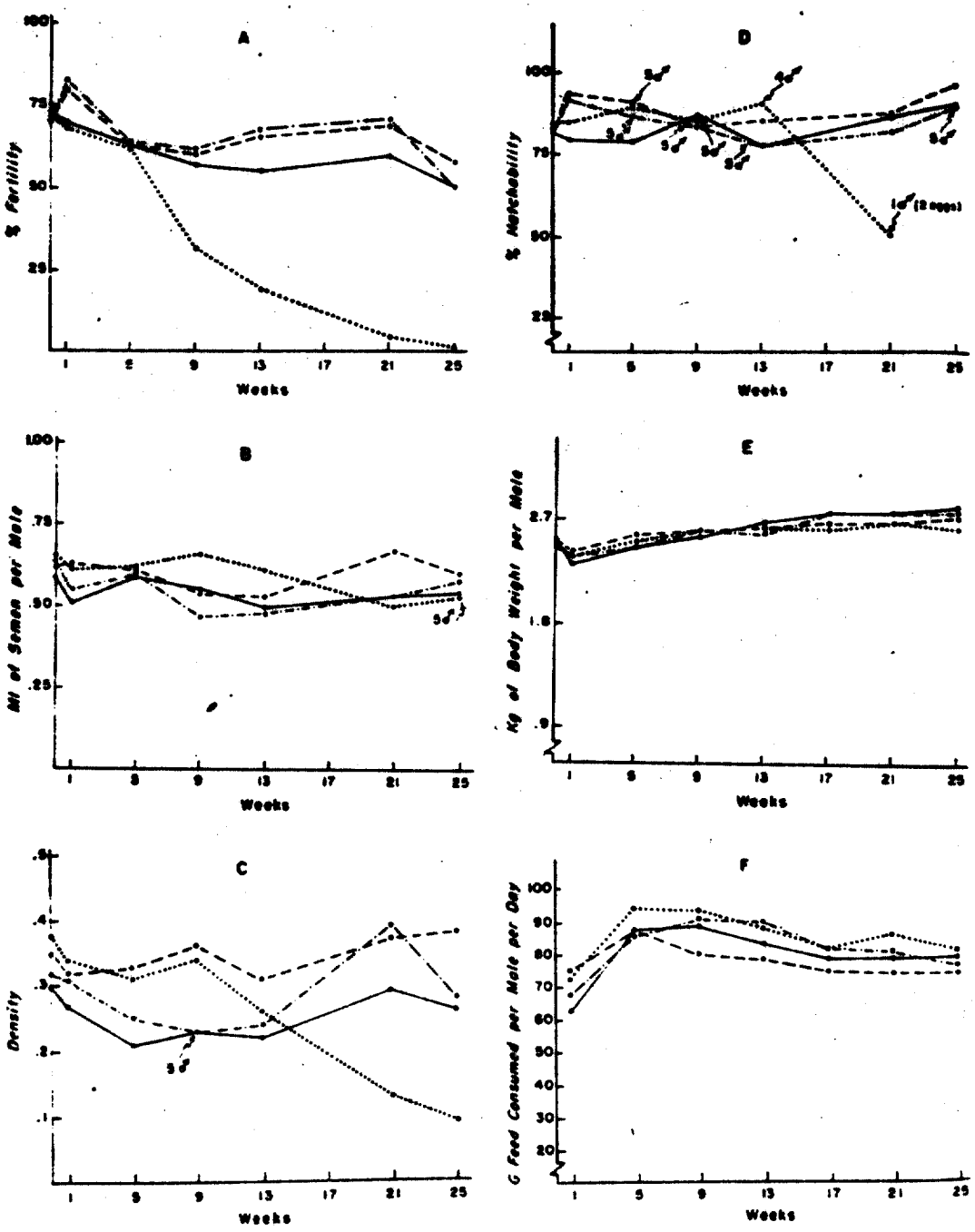

Fig. 1 Influence of linoleic acid (LA), vitamin E (VE) and ethoxyquin (EQ) on (A) fertilizing capacity, (B) volume and (C) concentration of semen; (D) hatchability; (E) body weights and (F) feed consumption in adult male chickens. Legend: low LA-VE-EQ – – –; high LA, low VE-EQ . . . . .; high LA-VE, low EQ ——; high LA-EQ, low VE —·—·—. Numerals indicate number of males from which data were derived when less than 6 males were involved.

TABLE 3

Effect of increasing volume and number of sperm inseminated per hen on fertilizing capacity of males fed diets high in linoleic acid with and without added vitamin E

Treatment	No. males	Volume inseminated	Optical density	Sperm index [1]	Fertility	
					All	Paired
		ml			%	%
		26th Week				
High linoleic acid — low vitamin E (diet 2)	3	0.06(1) [2]	0.046	2.76	0	0
	4	0.06(2)	0.161	9.66	0	0
	8	0.1	—	—	0	—
	12	0.1	0.061	—	0	—
	13	0.07(3)	0.174	12.18	0	0
	17	—	—	—	—	—
Avg			0.111	8.20	0	0
High linoleic acid and vitamin E (diet 3)	7	0.05(1)	0.051	2.55	92.9	92.9
	11	0.04	0.328	—	86.7	—
	15	0.03	0.377	—	92.9	—
	16	0.03	0.420	—	92.3	—
	21	0.04(3)	0.268	10.72	75.0	75.0
	23	0.05(2)	0.215	10.75	23.1	23.1
Avg			0.277	8.01	77.2	63.7
		27th Week				
High linoleic acid — low vitamin E (diet 2)	3	0.1(4)	0.102	10.20	0	0
	4	—	—	—	—	—
	8	0.1(5)	0.076	7.60	0	0
	12	0.1	0.022	—	0	—
	13	0.1(6)	0.215	21.50	0	0
	17	—	—	—	—	—
Avg			0.104	13.10	0	0
High linoleic acid and vitamin E (diet 3)	7	0.03(5)	0.208	6.24	93.8	93.8
	11	0.03(4)	0.319	9.57	100.0	100.0
	15	0.03(6)	0.456	13.68	69.2	69.2
	16	0.03	0.398	—	58.3	—
	21	0.03	0.552	—	100.0	—
	23	0.03	0.456	—	100.0	—
Avg			0.387	9.83	86.9	87.7

[1] To reflect relative numbers of sperm used for insemination, obtained by multiplying the volume of semen used × optical density × 1000.
[2] Numbers in parentheses represent paired males.

min E and ethoxyquin; however, no clear-cut treatment effects on hatchability were observed to 13 weeks and the 21-week figure is based on only one male and 2 fertile eggs, since only one of the 6 males fertilized any eggs at 21 weeks. No differences were observed between treatments for body weight (fig. 1E) and feed consumption (fig. 1F).

No differences in testes size between treatments were noted. Average weight of both testes per male for diets 1, 2, 3 and 4 were 19.3 ± 3.7, 20.5 ± 12.5, 17.3 ± 6.6 and 16.7 ± 2.8 g, respectively.

Increasing the volume of semen used from the group of males fed at the high level of linoleic acid without added vitamin E (diet 2) so that the total number of sperm (sperm index) from this group and the group receiving added vitamin E (diet 3) were more comparable, failed to improve the fertility of eggs during the 26th and 27th week of the experiment (table 3). In all instances where increased amounts of semen were used, fertility was zero from all males fed the diet containing the high level of linoleic acid without vitamin E, indicating that this diet ad-

versely affected the fertilizing capacity of the sperm.

These observations show that a diet containing a high level of linoleic acid in the absence of supplemental levels of vitamin E and ethoxyquin has an adverse effect on fertility of adult male chickens. These observations are interesting in view of those of Machlin et al. (2) showing that high linoleic acid without vitamin E and ethoxyquin resulted in decreased fertility of hens. Contrary to the work of Adamstone and Card (1), feeding a diet to males deficient in vitamin E without a high level of linoleic acid did not adversely affect fertilizing capacity. It is recognized, however, that this experiment did not extend over a 2-year period as did their experiment. These results suggest that vitamin E like ethoxyquin serves primarily as a biological antioxidant. Of particular interest is the observation that semen volume was not adversely affected, whereas sperm concentration was. Furthermore, increasing the number of sperm used for insemination failed to exert any beneficial effect on percentage of eggs fertilized, indicating that feeding rations high in linoleic acid and low in vitamin E and ethoxyquin not only reduced the numbers of sperm but also the fertilizing capacity of those that were produced.

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THE INFLUENCE OF ALPHA-TOCOPHEROL ON THE BASAL METABOLISM

W. Baroni - G. Casa

In 1938, Karel and Todd succeeded in chemically identifying and synthesizing the vitamin E discovered by Evans and Bishop, isolating it from the oil of wheat germ. Vitamin E is very widespread in nature; especially rich in it are lettuce and the oil of wheat germ. In the animal organism it is contained in notable quantities in the muscles and in the liver; according to Evans and Burr, the placenta and the hypophysis would contain the highest percentage of it. The daily requirement in man appears to be .20 mg per kg (about 12 mg in an adult male). This vitamin, which was kept in earlier times specifically for the treatment of certain disfunctions such as female sterility or azoospermia, now has found useful application in many illnesses, and among the most disparate. Therapeutic information on diabetes mellitus is based on the results attained by Williams, Butturini and Vogelsang, and many others, who ascertained that α-tocopherol acetate strengthens the action of insulin, improves the vascularization of the sclerotic pancreas, and that an improved conveying of blood to the islands of Langherans results in a diminution of hyalinization and of fibrosis, with an augmentation of its specific activity.

Vitamin E finds a vast field of application in cardiopathies. F. Shute, V.F. Shute, and Vogelsang affirmed its efficacy in anginas and hypertensive myocardiosclerosis; Mantero, Rindi and Trozzi in myocardial infarction. Butturini defines as favorable the results of tocopherolic therapy in hypertension, myocardosis and post-infectious myocarditis, Bürger's disease and Dupuytren's disease. Good results were obtained in cases of thrombophlebitis of the limbs and cerebral thrombosis (Shute, Vogelsang, Castagna, Impallomeni, Kay, Hutton, Balla, Ockner.).

The theory that vitamin E acts in a beneficial way on the connective tissues, leading to the neo-formation of collagenous tissues and activating the hyalurodinasal fermentum which regulates the nutritive exchanges is supported by many authorities (Steinberg in the case of primary fibrosis, Burgess and Pritchard in lupus erythematosus, Katz, Galzi in induratio penis plastica). Ballanti has established experimentally that α-tocopherol acetate is capable of preventing peritoneal frictions; favorable practical results were also noted in the treatment of hemorraghic syndromes (Prosperi, Tropeano, Vogelsang, Shute); in several hepatopathies there were also good results (Schwarz in catarrhal jaundice and calculoses, Butturini in acute benign hepatopathy). Biagini, through experimental work, also confirmed

that vitamin E accelerates a considerable repair of the liver in rats subjected to partial hepatectomy; such regeneration occurs to a large extent through hyperplasia and the successive transformation of hystiocytic elements. Quite a few authors report probative therapeutic results in amyotrophic lateral sclerosis (Wechsler, Mayer, Sabotka); the process of cure of cutaneous wounds would also be hastened by vitamin E (Marchesi, Pregreffi and Bartolomucci). Bottiglioni and Sturani affirm that vitamin E possesses a constant and well-manifested spleno-reductive action; Ziesche Hermogenes claims to have achieved better successes with vitamin E in the treatment of Erb's myo-dystrophy than those achieved with glycocoll. Proceeding with systematic research, Kavinsky confirmed the results obtained by preceding researchers, according to which vitamin E represents a safe and effective medicine in cases in which the estrogens are contra-indicated. Malhuret and Wallerand obtained good results with 7 of 9 boys affected with enuresis and treated with α-tocopherol.

From this quick bibliographical survey it is seen that vitamin E has come to be of much use in medical practice because it is endowed, beyond a specific vitamin action, with a high reducing power which renders it active beyond any of its vitamin characteristics. Heinsen defines vitamin E as a substance which normalizes the function of the hypophysis, and considers it above all the "vitamin of the metabolism", rather than the vitamin of sterility. According to the above-cited authors, vitamin E would hasten the anterior lobe of the hypophysis to a greater secretion of gonadotropic hormones, and would likewise immediately stimulate all the diencephalic-hypophysial functions, primarily the regulation of the entire metabolism. That vitamin E is a principal regulator of certain phases of the basal metabolism is a fact that has been verified beyond doubt for some years, but only recently have some of these scientific acquisitions been able to become concretized in precise therapeutical applications.

Butturini, proceeding from the considerations of Bomskow and von Kaulla, which denied a probable relation between vitamin E and carbohydrate exchange, expanded the argument by completing the research with the study of creatinic exchange in rats with avitaminosis E. Heinsen also, studying the glycemic metabolism in 15 subjects with alterations of the glycidic metabolism (with diencephalo-hypophysial disorders), concludes that vitamin E leads to a clear reularization of the glycemia from the stress of insulin (Radoslaw's curve) and concludes that α-tocopherol is a regulator of prehypophysial activity. Studies concerning the influence of vitamin E on the basal metabolism are not many, and nearly all conducted on animals. Victor and Madsen noted a marked increase (from 200% to 400%) in the consumption of oxygen in rabbits kept on diets lacking in vitamin E, whereupon dystrophic lesions appeared in the skeletal muscles. Kaunitz and Pappenheimer, who studied the consumption of oxygen in rats on diets deficient in vitamin E and compared this consumption with that of rats kept on the same diet but with

the addition of vitamin E, found an increase in said consumption in the rats deprived of the vitamin. Singer and Barrié verified alterations in the hyperplastic sense of the thyroid in rats deprived of the E factor. Pappenheimer studied the consumption of oxygen in muscles cultivated in vitro, and saw a reduction in the consumption of O_2 upon the addition of vitamin E to the nutritive liquid. Confirmation of this data is found in the observations of Veheler and Perkinson, who affirm that while hyperthyroidism increases the need for vitamin E, and while thyroxine provokes an increase in the consumption of oxygen, vitamin E often produces a decrease in the consumption of oxygen in the muscle subjected to the action of thyroxine, as is the case in muscle in a state of avitaminosis E; thus the vitamin also produces in the organism a decrease in the creatinuria provoked by the administration of thyroxine. If in some experiments on man Roncollo and Pellegrini affirm that the use of vitamin E induced a slight increase in the basal metabolism, Hottinger, Hickman, Harris, Borman and Heinsen a decrease in the basal metabolism so marked and constant that it led them to believe they could find in vitamin E the possibilit of an optional treatment of hyperthyroidism.

In fact, after the administration of vitamin E orally at a dose of 60 mg a day for three days, Heinsen observed a decrease in the formerly normal basal metabolism, and this decrease was even far more pronounced after the suspension of the administration; in cases of parenteral administration of an oily solution of vitamin E, he did not observe any manifest variations in the exchange of oxygen; however, the quantities of vitamin were at a level lower than than in the former doses. The author considers it possible that the lack of response may be due to an insufficient utilization of the vitamin E introduced parenterally as compared to that introduced orally. From the investigations already reported, we see evidence of the narrow relationship between vitamin E and endocrine glands, in particular the hypophysis, the thyroid and the gonads. Interesting in this respect is the research of Barrié, who observed how hypophysectomy in pregnancy causes miscarriage and the cessation of lactation, whereas if only the neurohypophysis is removed, the pregnancy continues just the same; the author shows how total ablation of the gland evokes a picture analogous to those seen in cases of protracted vitamin E deficiency, which suggests a relation between the two phenomena which might with all probability lead to acknowledgement of the fact that said vitamin may be necessary for the normal functioning of the adenohyphysis. Robecchi also affirms the possibility of vitamin E's representing the substance basic to the function of the anterior hypophysis and the prehypophysial gonadotropic hormones, with which it shares an identical action. On the other hand, a modified activity of the prehypophysis can also sustain itself on the basis of the modifications which intervene during the period of deficiency in other glands subordinate to this one, and especially in the thyroid, modifications which some see explained in a regressive sense (Rowlands, Singer,

Tomutti, Barrié, Anderson), while in the opinion of others they are manifested in hyperplasia of the organ (Emerson, Evans, Mason, Telfort, Bryan).

The bibliographical synthesis already presented demonstrates how vitamin E is endowed with a complex of pharmacological actions which definitely exceed those which in the recent past constituted its principal clinico-therapeutic indications. The literature on the argument recalls attention to the possible use of the tocopherols in several alterations of the metabolism, but since, as it appears from the premises, studies in this field are exceedingly scarce (to our knowledge only Heinsen has dealt with it), we felt a desire to study the behavior of the basal metabolism under the influence of α-tocopherol in subjects of normal metabolism and excessive exchange of oxygen. The observations were conducted among three groups of patients. In the first group, composed of 10 subjects, the basal metabolism was determined before and an hour after the parenteral administration of 300 mg of vitamin E. In the second group, composed of 6 subjects, our inquiries were carried out before and after a treatment prolonged over a week-long period, with daily doses of 600 mg of α-tocopherol, half of which was administered intramuscularly in an oily solution, and half orally. In the third group, composed of patients with elevated basal metabolisms, the metabolism was checked after 4 weeks of vitamin treatment in doses of 300 mg, administered intramuscularly on alternate days.

If we observe the result of the completed analysis, we can claim that from now on there can exist no doubt concerning the action of vitamin E on the regulation of oxygen exchange. In fact, as is shown by the data reported schematically in table no. 1 (which reports the metabolic values of the 10 subjects in whom the basal metabolism was studied before and after a single injection of 300 mg of α-tocopherol), the administration of vitamin E exercised an evident decreasing effect on the basal metabolism. In seven of the ten subjects, the decrease was so marked as to surpass quite signally the margin of physiological oscillation. In 2 cases, on the other hands, a slight increase in the metabolism was achieved; in the remainder there was no change.

In the second group of patients, after a daily treatment of 600 mg of α-tocopherol (300 mg administrated intramuscularly and 300 mg orally), there occurred a more constant reduction in the exchange of oxygen, with the exception of the first case, in which the basal value remained invariable.

In the third group of patients, where the administration was of larger quantities and was more prolonged, we obtained the clearest and most constant results: in all, in fact, the basal metabolism, which resulted in exceeding the norm, immediately underwent a definite decrease.

On the whole therefore, on the basis of our research, we can conclude that vitamin E almost constantly causes a sensitive decrease of the basal metabolism, and that the most evident results are obtained through more prolonged treatments.

In our opinion, the mode of administration has thus far given no evidence of any particular significance. In fact, our study offers a useful practical contribution to the question of the activity of α-tocopherol as related to the mode of administration.

Tabella n. 1.

Caso n.	M B	Met. dopo 1 ora dalla introduzione di 300 mg. di vit. E
1	+ 21	+ 8
2	+ 46	+ 30
3	+ 38	+ 38
4	+ 31	+ 37
5	+ 47	+ 32
6	+ 31	+ 11
7	2	± 0
8	25	+ 11
9	+ 2	+ 11
10	+ 14	± 0

Tabella n. 2.

Caso n.	M B	Met. dopo 6 giorni di trattamento con 300 mg. di vit. E
1	+ 2	+ 2
2	+ 12	+ 6
3	+ 19	+ 8
4	+ 16	+ 13
5	+ 21	+ 16
6	+ 30	+ 22

Tabella n. 3.

Caso n.	M B	Met. dopo [illegible] giorni di trattam. con [illegible] mg. di vit. E
1	+ 26	+ 11
2	+ 58	+ 32
3	+ [illegible]	+ 47
4	+ 70	+ 39
5	47	+ 24
6	+ 43	+ 26
7	62	+ 51

[illegible] Evien forte s, fiale e [illegible]

Key:
1.= metabolism after one hour following introduction of 300 mg of vitamin E.
2.= metabolism after 6 days of treatment with 600 mg vitamin E.
3.= metabolism after 30 days of treatment with 300 mg vitamin E.

Demole noted that vitamin E administered parenterally (through subcutaneous membrane and intramuscles) had an effect five times lower than that administered orally, and explains this fact as an incomplete absorption of the oily substance. Cerloczy and Bartels agree with the above-cited author, while Prosperi and Baronchelli affirm that vitamin E is active no matter how it is administered, whether orally or intramuscularly or intravenously: the results which we obtained support the thesis of these latter authors, leading us to conclude that

the vitamin conserves those properties which are active upon the metabolism whatever may be the mode of administration.

Vitamin E when swallowed, be it naturally, mixed with food, or experimentally in concentrated solutions, is absorbed rapidly in as much as it is true that a single dose of it leads, in the space of 5 to 6 hours, to a clear elevation of the rate in the blood; in 24 hours after the administration, the rate reaches physiological values. (J. Couperus) According to some authors, (Hines-Matill) the administration of the vitamin is followed by total absorption only when it is administer in doses which are physiologically necessary, while the excess would not be completely utilized and would be eliminated with the feces. However, other authors are not of the same opinion; nonetheless, there is no sufficient evidence to support the supposition that in man administered doses of 600 mg (inasmuch as this quantity is greatly superior to the requirement) need necessarily lead to a useless dissipation of the administered vitamin in the feces. Since neither tocopherolics nor quinonic products are ordinarily found in the urine (Hines-Mattill), it is conceivable that the processes of destruction, probably through successive demolitions, may be pushed as far as to obtain products which have a relation only to some parts of the complex original form of tocopherol; the absorption of the vitamin depends in part on fat and bile, with a reserve accumulation in some organs, such as the prehypophysis, the testicles, the pancreas, and the placenta. Elimination occurs above all through the feces and is in general slow: in fact, after an administration of 50 mg of vitamin E, the vitamin reaches its maximum percentual in the adipose tissue after 7 or 10 days, with a return to normal values only after about two months.

The hypotheses hitherto advanced concerning the mechanism of the action of the tocopherols on the exchange of oxygen are varied and sometimes contrasting. One of the more accredited opinions maintains that the fundamental action is that of reducing oxidizations in all the tissues of the body, especially those of the skeletal muscles. This hypothesis is supported by the experiments of Hove, Hickman and Harris, who observed a greater resistance to hypooxyhemia in rats treated with vitamin E than in control rats. According to Schaffer, Juhasz and Adamstone, the tocopherols exercise a definite influence on cell division and proliferation and are, according to Mason, necessary for the synthesis of the nuclear chromatin and for the maintenance of its physicochemical state. More recent research demonstrates that vitamin E is involved in the basal metabolism of substances containing the phenanthrenic nucleus, esterol, androgens, estrogens and progesterone (Adamstone). The fact that the tocopherols are connected with the absorption and utilization of the lipides and the products of their intermediate metabolism has been asserted by Mason, Ravina, Pichet and Meldolesi, who affirm that the tocopherols can inhibit the proteolithic substances of the serum which, by their activity, are opposed to the normal metabolism of proteins

and their transformation into muscular tissue.

Moore also affirms that vitamin E intervenes in the metabolism of proteins, and recently Müller, having achieved an inhibition of hyaluronidase with esters of tocopherol, stated his belief that an important function of vitamin E may consist in the diminution of the activity of hyaluronidase. From results which he obtained, Grifa points out that vitamin E acts as an inhibitor of hyaluronidase in vivo and in vitro, and increases moreover the anti-hyaluronidasal power of the serum, and on the basis of his research, the author is inclined to believe that an important function of vitamin E and its derivatives is that of regulating the activity of the hyaluronidase in man. For Heinsen, the point of attack of vitamin E is on the diencephalic-hypophysial system, so that he considers it the "regulator of the hypophysial function". In the conclusion of his "Critical and Pathogenetic Revision of Vitamin E Therapy", Butturini affirms that vitamin E can explain its therapeutic action on organs as well as different systems with a unique fundamental mechanism, i.e., that of intervening in the processes of intercellular oxide-reduction and phosphoralization of carbohydrates and fats, as well as indirectly the exchange of proteins. We maintain that vitamin E exercises a complex influence on the organism, an influence that embraces the diverse hypotheses outlined with a single operational thread, which can be none other than the regulation of the system of the hypothalamic centers.

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On the Stimulating and Inhibiting Effect of Vitamin E on the Diuresis, and its Dependence on the Single Dose

by
H. Becker-Freyseng

(Entered on June 23, 1951)

From the works of Heinsen we know of the effect of vitamin E on water retention, regarding its dependence on the the starting endocrine condition of the patient, and on the total dose. The observation that an excessive, as well as an inhibited diuresis can be normalized by a protracted administration of vitamin E, led the above-mentioned doctor to speak of a regulator of the hypophysial function. Moreover, he noted that, in healthy individuals, an increase in the diuresis at first occurred, but that upon continuation of the administration of the vitamin, a decrease set in; this the doctor attributed to either the exhaustion of the hypophysis or to inhibition by increased formation of sex hormones. Heinsen and Bormann do not consider a single dose of vitamin E effective, but believe rather, "that vitamin E apparently does not produce any effects until it is given in a certain dosage, and in short-term experiments it reveals no effect on the metabolic process after a single parenteral dose."

In contrast to this, clinical examinations of patients with oliguria due to diencephalic hypophysis revealed a definite increase in the diuresis upon the application of the Volhard water experiment following an injection of evion, that is, after 30 mg of α-tocopherol acetate. Even more surprising was a second finding, which revealed that in the same patients, injected with 60 mg, a lesser excretion, sometimes even one which in contrast to normal diuresis was extremely inhibited, set in. In order to eliminate the effect of the total dose, patients H.B. and M.Sch. were given doses in alternating sequence (see table).

1. 2.

Patienten	Einfacher Wassertag	30 mg Vitamin E	60 mg Vitamin E
K. Bö.	449	1485	1121
H. B.	1160	1314	800
M. Sch.	893	910	123

3. 4-Std-Ausscheidung nach 1500 cm³ Tee.

Key:
1.= patients
2.= plain water day
3.= 4-hour excretion after 1500 cm^2 of tea

This discovery of a paradoxic vitamin E action dependent on the dose seems to me to be of fundamental significance for

the therapuetic use of vitamin E, as well as for the theory of vitamin E action. Today, single doses of vitamin E which amount to between 40 and 70 times the effective quantity of 30 mg are being used in part for therapeutic purposes. The supposition that the effect of vitamin E is based on the equalization of equalisation of vitamin E deficiency in the midbrain system might, in view of the above finding, require further amplification. A detailed representation of the finding and its description will be given elsewhere.

Summary. Beyond the hitherto known action of vitamin E, attention is drawn to the effectiveness of small single doses (30 mg of α-tocopherol acetate = 1.0 evion*) on the diuresis, as well as to the reversal of effect dependent only upon the single dose. While 30 mg of vitamin E increase the diuresis in patients with oliguria due to diencephalic hypophysis, 60 mg of the same exercise an inhibiting influence upon the diuresis.

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* I respectfully extend my thanks to the firm of E. Merck, Darmstadt, for the use of their preparation, evion.

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38 (3/4): 328–344

Labelled Tocopherols in Man

Intestinal Absorption and Thoracic-Duct Lymph Transport of *dl*-α-Tocopheryl-3,4-$^{14}C_2$ Acetate *dl*-α-Tocopheramine-3,4-$^{14}C_2$ *dl*-α-Tocopherol-(5-methyl-^{3}H) and N-(methyl-^{3}H)-*dl*-γ-Tocopheramine.

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Summary: The intestinal absorption of dl-α-tocopheryl-3,4-$^{14}C_2$ acetate, dl-α-tocopheramine-3,4-$^{14}C_2$, dl-α-tocopherol-(5-methyl-^{3}H) and N-(methyl-^{3}H)-dl-γ-tocopheramine has been studied in man. The labelled compounds were administered in a formula meal to patients with cannulated thoracic ducts. Serial samples of lymph were collected and the lipids were extracted.

The total recovery of the administered radioactivity of tocopherol and derivatives in the lymph lipids ranged from 21.3 to 90.9 per cent in 24 hours. The greatest recovery was found after administering N-(methyl-^{3}H)-dl-γ-tocopheramine, followed in order by dl-α-tocopheramine-3,4-$^{14}C_2$, dl-α-tocopheryl-3,4-$^{14}C_2$ acetate and dl-α-tocopherol-(5-methyl-^{3}H). The major part of the recovered radioactivity was located in the chylomicrons. Absorption of radioactivity into the lymph occurred mainly between 2 and 8 hours after administration.

The labelled material in the lymph was separated from triglycerides, cholesterol esters and phospholipids by chromatograhy of the lymph lipids on Sephadex LH 20. By means of adsorbant thin-layer chromatography and reversed-phase thin-layer chromatography most of the labelled material recovered in the lymph was identified as being identical with the administered labelled compound when this was tocopherol or tocopheramine; when tocopheryl acetate was given it was recovered in the lymph as tocopherol.

The results show that tocopherols are absorbed via the lymphatic pathway in man, that tocopherol and tocopheramine enter the thoracic-duct lymph intact and that tocopheryl acetate is split and recovered as tocopherol.

It is suggested that the low intestinal absorption of tocopherol and tocopheryl acetate might account for the deficieny of vitamin E associated with a diet enriched with polyunsaturated fatty acids.

In patients with biliary obstruction the lymph contained only minute quantities of radioactivity.

In 1920 Matill and Conklin noticed that disturbances in reproduction occurred when rats were fed on a certain milk diet. This observation awakened an interest in the relation between fertility and nutrition, and on this subject Evans and Bishop published a series of papers. In 1922 they wrote «On the existence of a hitherto unrecognized dietary factor essential for the reproduction» [13]. First called «vitamin X» or «antisterility vitamin», this dietary factor later became known as vitamin E. In 1936 Evans, Emerson and Emerson [14] isolated from wheat germ oil two forms of vitamin E and these were called tocopherols. The Emersons isolated the allophanates of α-, β- and γ-tocopherol from a number of sources. Vitamin E was first synthesized by Karrer and Isler in 1938.

Research on the metabolism and function of the fat-soluble vitamins was to some extent delayed on account of the rapid progress in research on the water-soluble vitamins. «Whereas we know the ways in which many of the water-soluble vitamins participate in the metabolic processes and what function they fulfill in the cells, the metabolic role of the fat-soluble vitamins is at the most a matter of speculation» (Karrer [23]). With regard to vitamin E another problem has been the difficulty of finding overt signs of deficiency in man, such as xerophtalmia, rickets and haemorrhagic disease in vitamin A, D and K deficiency, respectively [19].

Since the discovery of the dietary effect of polyunsaturated fatty acids on the serum cholesterol level in man the antioxidative action of tocopherols has aroused new interest. In man the most extensive studies were those by Horwitt *et al.* [20]. Horwitt's investigations have clearly established that vitamin E deficiency can occur in the physically normal adult and that the dietary level of polyunsaturated fatty acids may be the principal determinant of the requirement

under normal dietary and environmental conditions. In animals many investigations involving vitamin E have disclosed various signs of deficiency [11, 26].

The effect of vitamin E, unlike that of other vitamins, is not strictly associated with a certain chemical structure. β-, γ- and δ-tocopherol, analogues of α-tocopherol, all have a biologic activity, which, however, is lower than that of α-tocopherol. Among the tocopheramines biologically active compounds have recently been found, some of them with an even greater activity than α-tocopherol [17].

Fundamental for the understanding of the metabolism of a compound is the knowledge of the mechanism of its absorption. Only a few experimental investigations on the intestinal absorption of vitamin E in man have been conducted, and apparently none on the lymph transport of vitamin E in man. The purpose of the present study was to examine the absorption of labelled tocopherols and tocopheramines *via* the lymphatic pathway in man, and to determine the form in which they are transported in the lymph. The study is a continuation of earlier ones on the transport of fat-soluble vitamins in the thoracic-duct lymph in man [3, 4, 6, 8, 18].

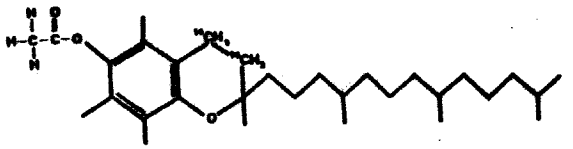
Methods

Labelled compounds. *dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate (specific activity 13.3 μc/mg), *dl*-α-tocopheramine-3,4-$^{14}C_2$ (specific activity 5.3 μc/mg), *dl*-α-tocopherol-(5-methyl-^{3}H) (specific activity 2150 μc/mg) and N-(methyl-^{3}H)-*dl*-γ-tocopheramine (specific activity 240 μc/mg) were stored under argon in sealed vials at –20° C until required, and were used without further purfication. Thin-layer chromatography of the labelled substances, performed as described below, indicated that, except in the case of *dl*-α-tocopheramine-3,4-$^{14}C_2$, at least 98 per cent of the radioactivity was associated with the tocopherol derivate. Thin layer chromatography of *dl*-α-tocopheramine showed that about 5 per cent of the radioactivity was associated with a more polar compound.

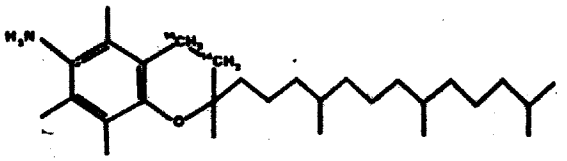
A survey of the labelled compounds used in this study is given in Table I and their formulae are shown in Figure 1.

Tab. I: The labelled compounds used and their specific activities

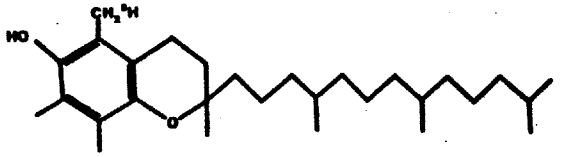
Isotope	Specific activity (μc/mg)
dl-α-tocopheryl-3,4-$^{14}C_2$ acetate	13.3
dl-α-tocopheramine-3,4-$^{14}C_2$	5.3
dl-α-tocopherol-(5-methyl-^{3}H)	2150
N-(methyl-^{3}H)-*dl*-γ-tocopheramine	240



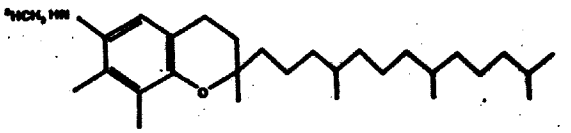
dl-α-tocopheryl-3,4-$^{14}C_2$ acetate
Spec. act. 13.3 μc/mg



dl-α-tocopheramine-3,4-$^{14}C_2$
Spec. act. 5.3 μc/mg



dl-α-tocopherol-(5-methyl-^{3}H)
Spec. act. 2150 μc/mg



N-(methyl-^{3}H)-*dl*-γ-tocopheramine
Spec. act. 240 μc/mg

Fig. 1: Labelled compounds.

Subjects and collection of lymph

The study was carried out in 2 patients, the morbid histories of whom are given in the following case reports. One of the patients IH-15, was submitted to only one examination, of *dl*-α-tocopheryl acetate, while in the other patient, EK-01, examinations were carried out with all 4 substances used, and also vitamins D_3 and K_1. The results obtained for these vitamins are published elsewhere [4, 5]. The surgical technique was identical with that described by Wrenn [33]. After local anaesthesia had been induced the thoracic duct was isolated near its entry into the venous system. A lymph fistula was produced by inserting a siliconized polyethylene catheter into the thoracic duct so as to collect all the lymph from this vessel.

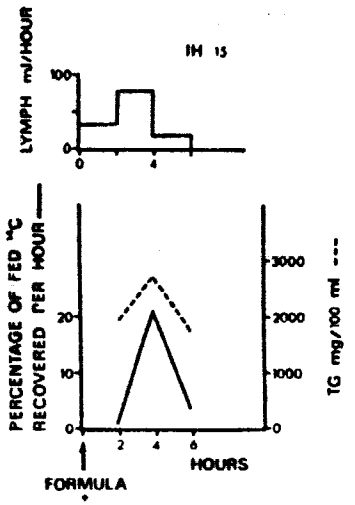


Fig. 2

dl-α-tocopheryl-3,4-$^{14}C_2$ acetate

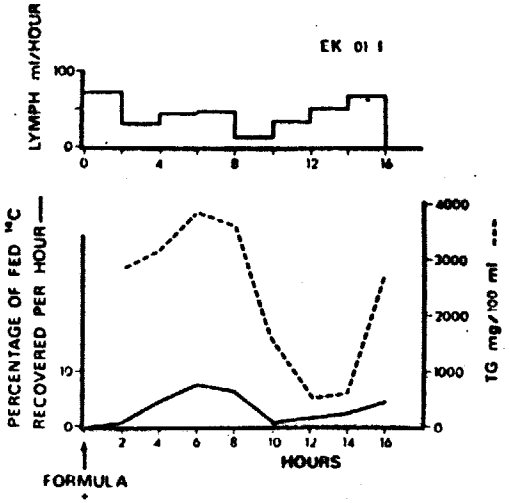


Fig. 3

dl-α-tocopheryl-3,4-$^{14}C_2$ acetate

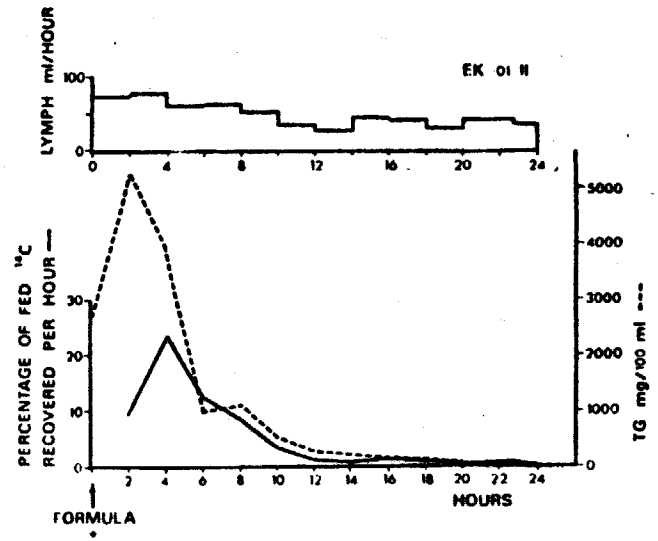


Fig. 4

dl-α-tocopheramine-3,4-$^{14}C_2$

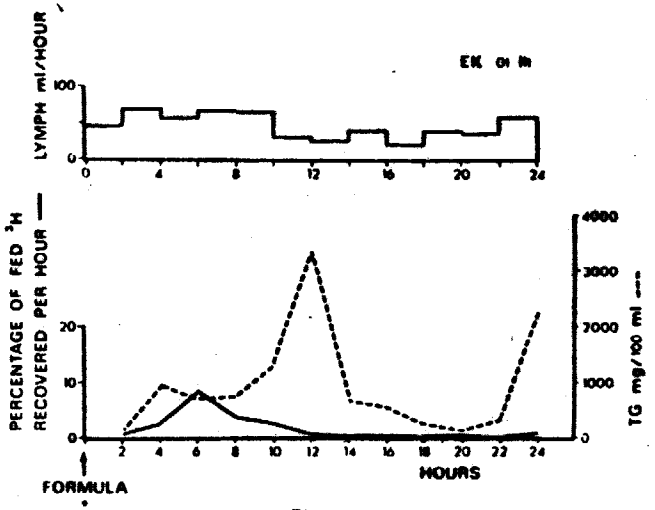


Fig. 5

dl-α-tocopherol-(5-methyl-^{3}H)

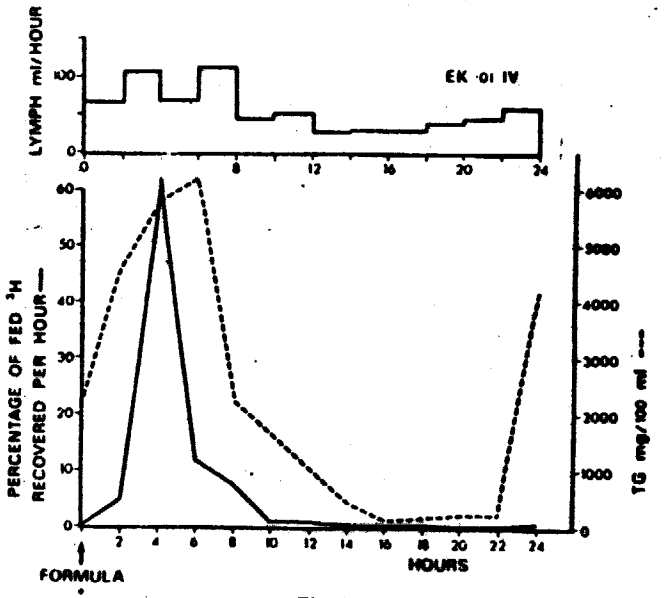


Fig. 6

N-(methyl-^{3}H)-*dl*-γ-tocopheramine

Figs. 2-6: Time course curves showing the appearance of radioactivity in lymph after administering labelled tocopherols in a test meal to 2 patients (5 examinations). Lymph volumes and concentration of triglycerides (TG) are recorded

The patient was fasted during the night prior to the operation (for about 14 hours). For the first 1-2 hours after the cannulation of the thoracic duct, the patient was allowed to drink fat-free liquids *ad libitum*. The lymph collected during this time was analysed with respect to electrolytes, protein, enzymes etc. The test substance was then given by mouth in 5 g of olive oil and 200-300 ml of skimmed milk. After this formula meal the normal hospital diet was resumed.

The lymph was conducted through the polyethylene catheter directly into glass bottles, to which a few drops of heparin solution had been added. The bottles were kept in a bedside refrigerator, so that the lymph could be collected constantly and stored in the dark and at low temperature (4° C). Throughout the period of examination the lymph was collected in 2-hour portions, the patient being kept under constant supervision. The lymph was replaced quantitatively with blood plasma or human albumin or, in the case of anaemia, with blood.

Besides the 2 patients mentioned in the case reports, another 3 were examined with the same technique. They were cases of biliary obstruction due to pancreatic carcinoma, and the study was performed to investigate the intestinal absorption of vitamin E in the absence of bile. The results of this study will be presented elsewhere and are mentioned here only as a preliminary communication [16].

Case reports

Patient IH-15. A woman aged 51 referred to the Department of Surgery for operation for gastric carcinoma.

Health previously good. For the last 6 months pain in the upper left part of the abdomen, with loss of weight, fatigue and malaise. Gastric radiographs disclosed carcinoma.

On admission, the general status was quite good, no palpable mass in the abdomen.

Laboratory tests: Bilirubin 0.3 mg/100 ml, alkaline phosphatase 2.1 (Bessey-Lowry), thymol 0.3; GOT 31, GPT 19 units/ml.

Biopsy specimens obtained from the left supraclavicular lymph-nodes showed no metastases. The thoracic duct was cannulated at the same time and the lymph was led off for a day. Plasma and albumin were given as substitute. The amounts of lymph collected during 6 hours after the formula meal is shown in Figure 2.

Some days later total gastrectomy was performed. The post-operative course was satisfactory and the patient was discharged about 3 weeks after the operation. Some months later she returned with pains, vomiting and emaciation and died a week later. Autopsy disclosed peritoneal carcinosis with ascites. The gall bladder was shrunken around a stone but the cystic duct and the common duct were free.

Patient EK-01. Woman aged 66 referred from Department of Internal Medicine to Department of Surgery for treatment for chronic lymphatic leukaemia with acute exacerbation. Apart from this, health and appetite previously good, able to eat any food, no gastrointestinal disorders or jaundice.

Lymphatic leukaemia discovered 5 years before admission. Extremely high lymphocyte count in peripheral blood. Cytostatic (Leukeran) immediately effective, and continued with good effect. No incapacitation.

During 6 months prior to admission lymphocyte count in peripheral blood had gradually increased despite administration of Leukeran. Simultaneously thrombocytopaenia and anaemia. It was decided to create a thoracic-duct fistula for lymphocytic depletion as treatment for lymphatic leukaemia.

On admission, general status good. No marked enlargement of lymph nodes on palpation, and physical examination disclosed no abnormal features. There was anaemia, with haemoglobin level 7.6 g/100 ml, erythrocytes 2.6 millions, thrombocytes 45,000 and leukocytes 420,000 with 1-3 per cent segment nucleated leukocytes and 97-99 per cent lymphoblasts.

Laboratory tests: Bilirubin 0.4 mg/100 ml, alkaline phosphatase 3.6 (Bessey-Lowry), thymol 1.2, GOT 18, GPT 13 units/ml.

A lymph fistula created by cannulation of thoracic duct. Simultaneously, biopsy specimens obtained from the left supraclavicular lymph nodes and from the right groin. Histologic examination showed changes suggestive of lymphatic leukaemia. Lymph drainage for 8 days followed by inexplicable arrest of lymph flow; catheter may have slipped from thoracic duct. The 9.5 l of lymph drained was substituted with blood, plasma and albumin. The amounts of lymph during the first 4 days are shown in figs. 3-6 and during all 8 days in fig. 7.

Drainage uncomplicated. Meanwhile, lymphocyte count decreased by about 50 per cent. On completion, patient returned to Department of Internal Medicine for further treatment.

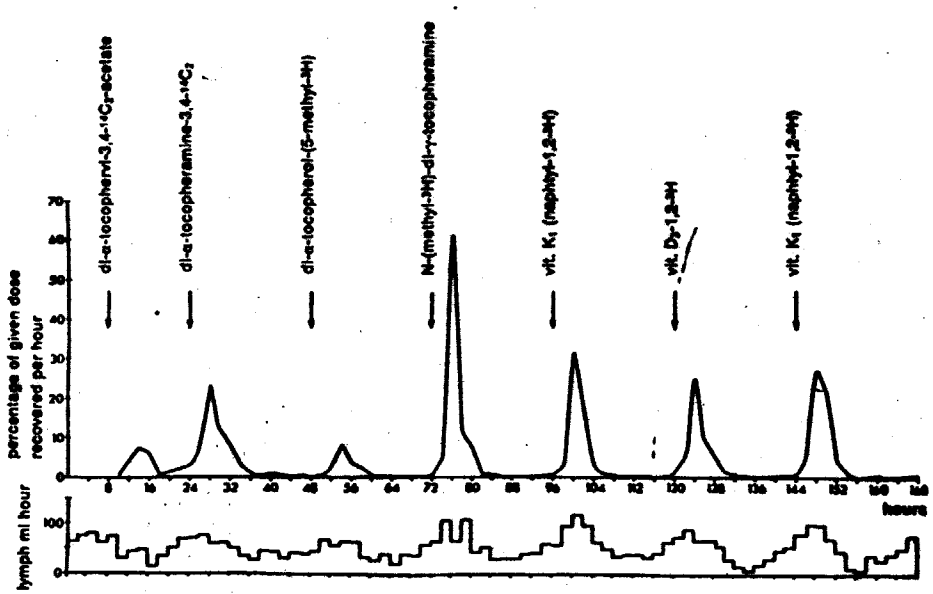


Fig. 7: Recovery of radioactivity in thoracic-duct lymph after oral administration of labelled tocopherols, vitamin K_1-^3H and vitamin D_3-^3H to patient EK-01. Time course curves of radioactivity in lymph and of lymph flow

Analytical procedures

The thoracic-duct lymph was collected in two-hour fractions by gravity drip directly into containers located in a bedside ice-box at 4° C. The volumes were recorded and the cells removed by low-speed centrifugation. A suitable fraction was extracted immediately with 20 volumes of chloroform-methanol, 2 : 1 (v/v), by a modification of the method of Folch, Lees and Stanley [15]. Chloroform extracts were evaporated to dryness, re-extracted and made up to volume with hexane. After adding scintillator solution as described earlier [4], aliquots were

counted in a Tri-Carb liquid scintillation spectrometer (Packard). Samples of the test meal were extracted in the same way and counted. The disintegration rate was calculated from efficiency data on the basis of known standards. The quenching was about the same for all the samples.

Triclyceride analysis was carried out on an extract of lymph by a modification of the method reported by Blankenhorn, Rouser and Weimer [1]. Chylomicron fractions were prepared from lymph samples by centrifugation in a MSE Superspeed 50 TC centrifuge at 25,000 g and 10° C. Lymph and chylomicron fractions were extracted with 20 volumes of chloroform-methanol, 2 : 1 (v/v), followed by evaporation and re-extraction with hexane, and stored under argon at –15° C.

For identification of the labelled material in the lymph about 100 mg of total lipid extracts from the various examinations was first chromatographed on 20 g of Sephadex LH 20 (Pharmacia, Uppsala, Sweden) using chloroform-methanol, 1 : 1 (v/v), by a modification of the technique evolved by Vihko [32], in which technique the various labelled tocopherols used were completely separated from the lymph triglycerides (Fig. 8). Portions of each column fraction were assayed for radioactivity. The radioactive fractions were combined, extracted, followed by evaporation and re-extraction with hexane.

For further identification, suitable amounts of carriers were added to the labelled material purified on Sephadex LH 20, and aliquots were first subjected

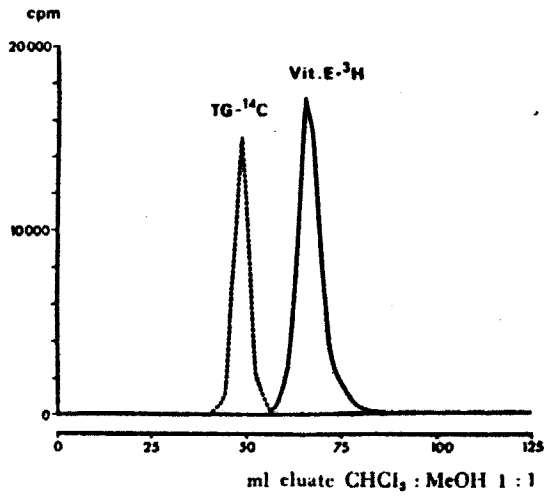


Fig. 8: Sephadex LH 20 separation of labelled tocopherols in human lymph lipids and ^{14}C-labelled triglycerides (TG ^{14}C) after feeding labelled tocopherols

to adsorption thin-layer chromatography on silica gel plates. The development of the chromatograms was performed in a tank lined on 3 sides with Whatman 3 MM filterpaper wetted with the solvent. The plates were developed in separate tanks containing benzene, cyclohexane-ether (4 : 1) or cyclohexane-ether-ethanol (16 : 4 : 3). Along with the analyses of the purified labelled material from the lymph lipids, mixtures of the labelled reference substances were also analysed. The radioactivity in various regions of each chromatogram was assayed after elution and expressed as percentage of the total radioactivity eluted.

The purified labelled material from the Sephadex LH 20 chromatography was also subjected to reversed-phase thin-layer chromatography on silica gel plates impregnated with paraffin oil using acetone-water, (90 : 10), saturated with paraffin oil as the mobile phase [9].

Results

Absorption of labelled tocopherols. The lymph flow through the thoracic duct fistulae and the percentage recovery of radioactivity in the lymph are given in figures 2-6.

In the first case, 1H-15 (Figure 2), the lymph was collected for only 6 hours after giving the formula meal with the radioactive substance, *dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate. During this time the lymph flow was quite as expected but then stopped abruptly, possibly because the catheter slipped out of the thoracic duct. During the 6 hour period 25 per cent of the administered radioactivity was recovered in the lymph.

In the other patient, EK-01 (Figures 3-6), the lymph drain was continued for 8 days. The 4 labelled compounds were given to the patient during the first 4 days, one each day. The first examination was started at 7 p.m. and the lymph was collected for 16 hours. The other 3 were begun at 11 a.m. and collection was continued for 24 hours. In these periods between 21.3 and 90.9 per cent of the administered radioactivity was recovered in the lymph. The greatest recovery, 90.9 per cent in 24 hours, was found for N-(methyl-^3H)-*dl*-γ-tocopheramine; next, in order, came *dl*-α-tocopheramine-3,4-$^{14}C_2$ (63.4 per cent in 24 hours), *dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate (28.5 per cent in 16 hours) and *dl*-α-tocopherol-(5-methyl-^3H) (21.3 per cent in 24 hours). When the tocopherol examinations were completed vitamin K_1-^3H was given on 2 occasions and vitamin D_3-^3H once (Blomstrand and Forsgren [4, 5]). The results of all 7 examinations are shown in figures 7 and 9 and table II.

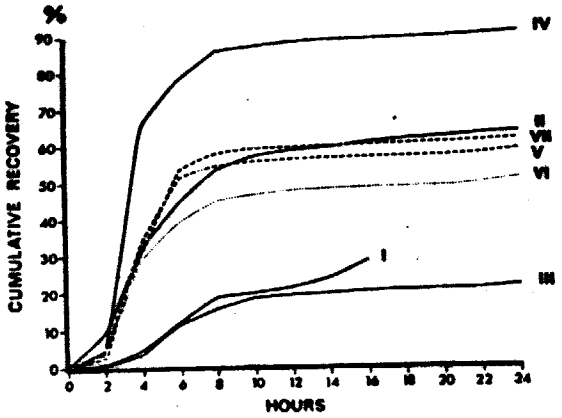


Fig. 9: Cumulative recovery of radioactivity in thoracic-duct lymph lipids after feeding labelled tocopherols, vitamin K_1 and vitamin D_3 to patient EK-01. I: *dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate; II: *dl*-α-tocopheramine-3,4-$^{14}C_2$; III: *dl*-α-tocopherol-(5-methyl-^{3}H); IV: N-(methyl-^{3}H)-*dl*-γ-tocopheramine; V and VII: Vitamin K_1 (naphtyl-1,2-^{3}H); VI: vitamin D_3-1,2-^{3}H

Tab. II: Recovery of radioactivity in the thoracic-duct lymph after oral administration of labelled fat-soluble vitamins (case EK-01)

		Isotope	Amount of isotope (μc)	Period lymph was collected after test meal (hours)	Recovery of radioactivity in lymph (per cent)
Expt. no.	I	*dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate .	20	16	28.5
	II	*dl*-α-tocopheramine-3,4-$^{14}C_2$	20	24	63.4
	III	*dl*-α-tocopherol-(5-methyl-^{3}H) ...	20	24	21.3
	IV	N-(methyl-^{3}H)-*dl*-γ-tocopheramine	20	24	90.9
	V	vitamin K_1 (naphtyl-1,2-^{3}H)	20	24	58.2
	VI	vitamin D_3-1,2-^{3}H	15	24	50.3
	VII	vitamin K_1 (naphtyl-1,2-^{3}H)	20	24	61.5

The recovery in all the tocopherol examinations of the 2 patients is summarized in figure 10 and table III.

The initial results for the patients with biliary obstruction indicate an extremely low recovery of tocopherols in the thoracic duct lymph (Figure 10). The lymph flow in one of them and the recovery of the labelled tocopherol are shown in figure 11. A detailed report of the patients with jaundice will be presented elsewhere.

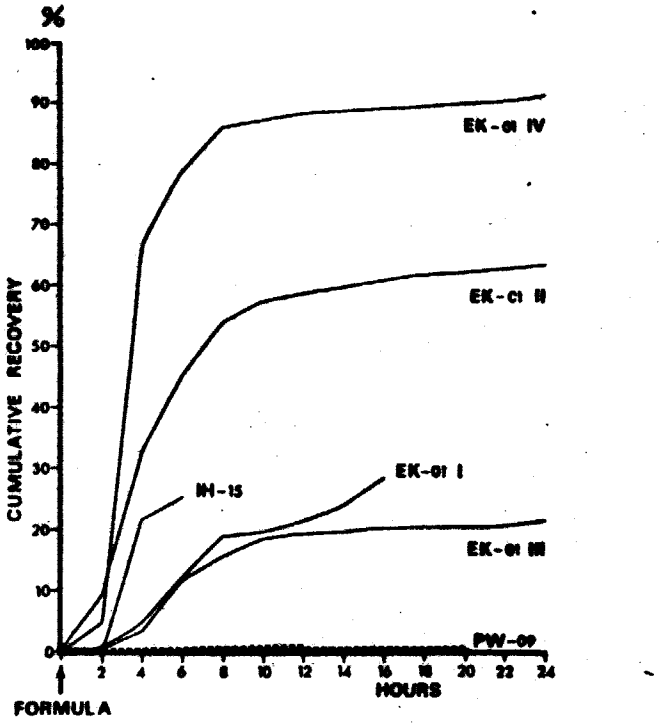
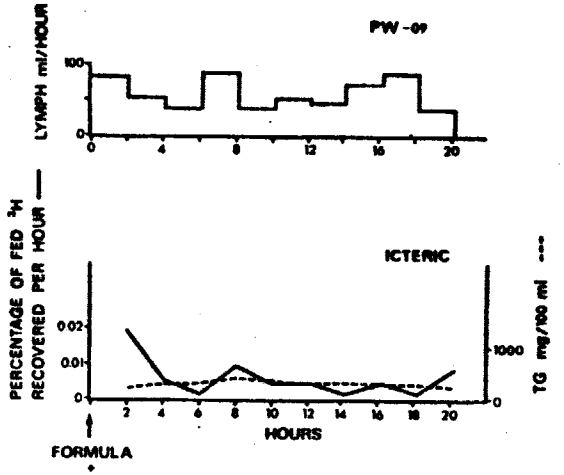


Fig. 10: Cumulative recovery of radioactivity in thoracic-duct lymph lipids after feeding labelled tocopherols in a test meal to 2 patients (5 examinations). *IH-15:* *dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate; *EK-01* I: *dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate; *EK-01* II: *dl*-α-tocopheramine-3,4-$^{14}C_2$; *EK-01* III: *dl*-α-tocopherol-(5-methyl-^{3}H); *EK-01* IV: N-(methyl-^{3}H)-*dl*-γ-tocopheramine. (The dotted curves near the zero-line refer to patient with biliary obstruction.)

Tab. III: Labelled tocopherols administered, time of lymph collection and total recovery of radioactivity

	Isotope	Amount of isotope (μc)	Amount of isotope (mg)	Collection time of lymph after test meal (hours)	Recovery of radioactivity in lymph (per cent)
Case IH-15	*dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate ...	20	1.3	6	25
EK-01	*dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate ...	20	1.3	16	28.5
	dl-α-tocopheramine-3,4-$^{14}C_2$	20	3.8	24	63.4
	dl-α-tocopherol-(5-methyl-^{3}H)	20	0.01	24	21.3
	N-(methyl-^{3}H)-*dl*-γ-tocopheramine	20	0.06	24	90.9



dl-α-tocopherol-(5-methyl-^{3}H)

Fig. 11: Thoracic-duct lymph volumes, recovery of radioactivity in lymph and lymph triglycerides (TG) in a patient with biliary obstruction fed labelled tocopherol

Nature of absorbed labelled material. In case EK-01 chylomicrons, isolated as described from the pooled samples representing the main peak of the absorbed radioactivity, contained about 77 per cent of the absorbed radioactivity after feeding labelled tocopheryl acetate. In the other examinations the relative amount of lymph radioactivity in the chylomicrons was not determined exactly. A similar distribution of radioactivity in human lymph has been found after feeding labelled β-carotene and vitamin A [8] and vitamin K_1-^{3}H [4]. In their study of the distribution of tocopherol in human serum lipoproteins McCormick *et al.* [27] found after oral administration of tocopherol that the newly absorbed tocopherol was first located in the chylomicrons and S_f 10-400 lipoprotein fractions.

Chromatography on Sephadex LH 20, in combination with thin-layer chromatography of the lymph lipid extract, showed the presence of free α-tocopherol in the lymph after feeding labelled α-tocopheryl acetate. The ester is apparently hydrolysed during absorption, as has been observed in the rat [34] and by means of faeces analyses in man [24, 25]. The recovery of radioactivity in the lymph was about the same whether tocopheryl acetate or tocopherol was given (Fig. 10), thus indicating about the same intestinal absorption of these 2 compounds. As shown by the adsorption and the reversed-phase thin-layer chromatograms, all

the radioactivity in the lymph lipids was associated with α-tocopherol. No significant amounts of tocopheryl quinone were detected [10].

After feeding *dl*-α-tocopheramine the greater part of the radioactivity in the lymph lipids was identified as unchanged *dl*-α-tocopheramine. In this chromatogram a small amount of radioactivity was located in the area of α-tocopherol, but it is conceivable that this was due to the ^{14}C-labelled *dl*-α-tocopheryl acetate fed on the previous day. The analyses thus indicate that the greater part of the administered *dl*-α-tocopheramine is absorbed intact. In a study of the metabolism of α-tocopheramine in the rat, Gloor *et al.* have obtained similar results [17].

With regard to the N-methyl-^{3}H-γ-tocopheramine both adsorption and reversed-phase thin-layer chromatography indicated that the greater part of the radioactivity in the lymph lipids was associated with unchanged N-methyl-^{3}H-γ-tocopheramine. Here, too, the chromatograms showed a small amount of radioactivity in the area of α-tocopherol, but this was too small for it to be confidently identified. Because ^{3}H-labelled α-tocopherol had been administered the previous day it was not possible to establish the origin of this radioactivity. After administering labelled *dl*-N-methyl-γ-tocopheramine to rats, Gloor *et al.* [17] were unable to find any radioactivity associated with α-tocopherol or α-tocopheryl quinone.

Discussion

It is apparent from the work of several investigators that a high percentage of a test dose of tocopherol is excreted in the faeces. Using chemical methods for determining tocopherol, Klatskin and Molander [25] found that 64 per cent of the daily intake in man is excreted in the faeces. Balance studies and determination of blood and liver levels of vitamin E in laboratory animals have also shown that its intestinal absorption is incomplete [12, 28, 30]. Knowledge of the rate at which a substance is absorbed is of great importance for the understanding of its metabolism. As regards vitamin E there are 2 complicating factors:

(1) As is the case for other fat-soluble vitamins the amount of vitamin E in food-stuffs is small compared with that of other lipids. In the investigation of the absorption and metabolism of vitamin E it is therefore necessary to use labelled compounds with a high specific activity so that the amounts required can be kept within physiologic limits. The specific activity of the labelled compounds used in this study varied (Table I) but it was always high enough to permit the use of small doses of the vitamin. In man with normal absorptive

mechanisms the daily requirement of α-tocopherol ranges from 10 to 30 mg [20]; the doses used in this investigation were well within the range.

(2) It has been shown in the rat that tocopherol is conveyed to the intestine by the bile, possibly constituting an entero-hepatic circulation [21, 29]. Partial confirmation of such a mechanism in man was obtained by Klatskin [24] from analysis of T-tube bile in subjects with obstructive jaundice. These revealed concentrations of tocopherol in bile comparable with those in plasma. Tentative results of an investigation of this problem at this Laboratory indicate that the bile is an important excretory medium for tocopherol in man [16]. Because of this it is impossible to draw any definitive conclusions from conventional balance experiments or blood analyses.

Cannulation of the thoracic duct enables a direct examination to be made of the amount of vitamin E absorbed from the intestine and transported in the lymph, and of the form in which it occurs. The problem of bile secretion of tocopherol is thus circumvented. Examination of the thoracic-duct lymph seems to be the only direct way of determining the intestinal absorption of vitamin E. A similar technique has recently been used to study the absorption of vitamin A and β-carotene (Blomstrand, Franksson and Werner [6]; Goodman, Blomstrand *et al.* [18]; Blomstrand and Werner [8]), vitamin D_3 (Blomstrand and Forsgren [3] and vitamin K_1 (Blomstrand and Forsgren [4]).

In a study of the intestinal absorption of ^{14}C-labelled α-tocopherol in rats with cannulated intestinal lymphatic ducts Johnson and Pover [22] found evidence that the main route of α-tocopherol absorption is *via* the intestinal lacteals and the chyle.

In all 4 examinations in case EK-01 the time course curves of the radioactivity in the lymph show that the greatest recovery was obtained 2-4 or 4-6 hours after administration of the vitamin (Figs. 3-6); after 8 hours 67-95 per cent of the total amount recovered had been found. In case IH-15 (Fig. 2), in whom only *dl*-α-tocopheryl acetate was administered, 25 per cent was recovered in 6 hours. These curves bear a close resemblance to those for the other fat-soluble vitamins mentioned above; they are also similar to those obtained in experiments on the rat and chick by Wiss, Bunnell and Gloor [34] in their study of absorption based on blood plasma levels and tocopherol concentration in the liver. The metabolic pathway of ^{14}C-labelled α-tocopherol has been examined in the rat by Sternberg and Pascoe-Dawson [31] in connection with metabolic studies in atherosclerosis; their results are in close agreement with those of Wiss, Bunnell and Gloor.

The total recovery of the labelled compounds given to patient EK-01 ranged

from 21.3 to 90.9 per cent (Table II). This large variation cannot have been due to a change in lymph flow from one examination to another, as this was found to be constant, displaying only normal diurnal variations (Fig. 7). It is therefore highly improbable that variations in recovery of labelled compounds could be due to variations in the flow of intestinal lymph.

Identification of the labelled compounds in the lymph by the various methods showed that they were identical with the administered tracers when these were *dl*-α-tocopherol, *dl*-α-tocopheramine or N-methyl-γ-tocopheramine. The fact that when tocopheryl acetate was given it was recovered in the lymph as tocopherol indicates that the ester is hydrolysed during absorption. The wide range for the recovery of the labelled compounds suggests a large variation in the absorption of the tocopherol derivatives from the intestine and their transport in the thoracic-duct lymph. The extremely high recovery of N-methyl-γ-tocopheramine, 90.9 per cent, is remarkable.

A possible source of error in all examinations in man performed by the method used in this study is that there might have been shunting of intestinal lymph past the thoracic-duct fistula *via* lymphatico-venous communications. The recovery of a perorally administered substance in the thoracic-duct lymph would then not provide a quantitative measure of the uptake by way of the lymph. The recovery of more than 90 per cent for one of the substances shows that in this patient there was no appreciable shunting of lymph past the fistula.

Although the 2 patients in this study had malignant diseases – chronic lymphatic leukaemia and gastric carcinoma – in neither of them were there any signs of impaired intestinal absorption. In the first examination of patient EK-01 the same labelled substance was administered as in the single examination of patient IH-15, namely *dl*-α-tocopheryl-3,4-$^{14}C_2$ acetate. There is a great resemblance between the recovery curves for these two examinations (Figs. 2, 3, 10).

The fact that the recoveries in the lymph of labelled tocopherol and tocopheryl acetate were of about the same magnitude, indicates a similar, fairly low, intestinal absorption. This is in contrast to the high intestinal absorption of polyunsaturated fatty acids found by a similar method in earlier investigations, when different labelled fatty acids were administered to patients [2, 7]. As mentioned above, it is established that a high level of polyunsaturated fatty acids in the diet increases the requirement of tocopherol. The difference in intestinal absorption of tocopherol and polyunsaturated fatty acids might account for the deficiency of vitamin E associated with a diet enriched with polyunsaturated fatty acids.

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The Effect of Cooking on α-Tocopherol in Vegetables

By V. H. Booth[1] and M. P. Bradford

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"Vitamin E" contents of vegetables have occasionally been reported, although usually only for raw materials. (See for example Harris, Quaife and Swanson [1950]; Herting, Kujawski, Ludwig and Harris [1958, 1961]; and McCance and Widdowson [1960].) Because it is desirable also to know the values for cooked vegetables, we have investigated the effect on α-tocopherol of cooking by boiling. The results presented in this paper suggest that the stability of α-tocopherol is similar to that of other fat-soluble vitamins.

Methods

Determination of tocopherol. The lipids were quickly extracted at room temperature with acetone and then with light petroleum. The extract was chromatographed in two dimensions on filter paper, treated first with $ZnCO_3$ and then with medicinal paraffin (Green, Marcinkiewicz and Watt, 1955). The α-tocopherol isolated thereby was measured with dipyridyl and ferric chloride. The procedure has been fully described elsewhere (Booth, 1963).

Cooking procedure. A pair of similar samples (0.3 to 3.0 g depending upon lipid content) were weighed. One sample was extracted at once. The other was placed inside a muslin bag, and the bag was then immersed in boiling water that already contained about 100 g of the vegetable. After 30 minutes the bag was removed, and the sample was extracted. Both extracts were then chromatographed.

This procedure obviated the taking of samples from a cooked mass with its complex problem of referring to the tocopherol value for uncooked material. The insertion of the small sample into a larger quantity of stewing vegetable also allowed practical cooking conditions to be simulated.

[1] Member of the scientific staff of the Agricultural Research Council.

Dry matter was calculated from the weights of a sample before and after heating for 5 hours at 100 °.

The effect of boiling for 30 minutes on the tocopherol in vegetables

Material	α-Tocopherol, p.p.m.* raw	cooked	Loss or gain, %	D.M.,** %
Brussels sprouts (*Brassica oleracea* cv)	4.4	4.1	−7	15.0
Brussels sprouts (*Brassica oleracea* cv)	8.7	8.6	−1	14.8
Cabbage, outer leaf (*Brassica oleracea*)	80	75.5	−6	20.5
Carrot (*Daucus carota*)	5.0	4.6	−8	9.8
Leek (*Allium porrum*)	3.9	4.1	+5	9.2
Yew (*Taxus baccata*)	310	295	−5	28.0
			Average −3.7	

* µg/g based on fresh weight. ** Dry matter.

Results

The results of the tests are summarized in the Table. Each result is the mean of triplicates. The α-tocopherol contents are expressed on a fresh weight basis, but dry matter contents are given so that tocopherol contents of the starting material may be computed on a dry matter basis if required. In brussels sprouts, carrots and leeks the samples were taken from the "edible part". The not usually eaten outer leaves of cabbage were used because they contain most of the tocopherol. Leaves of yew were tested because they represent a rich material.

Other leaves—nettle, ivy, clover—were also tested. They showed on average an apparent gain of 4 %, which is within the range of the results in the Table, but they are not mentioned therein because their stewing time was only 2 minutes.

(We have not found significant amounts of non-α tocopherols in common vegetables. The reports by other authors using less specific methods, of appreciable amounts in some materials, are being investigated.)

Conclusion

The average of the results in the Table was a loss of 3.7 % of the α-tocopherol. The coefficient of variation of one determination estimated from replicate determinations on many comparable vegetables, was ± 8.5 %: for the mean of triplicates this becomes ± 4.9 %. The observed loss is therefore of about the order of magnitude of experimental error. It is concluded that α-tocopherol,

which is insoluble in water, is not leached out by the cooking fluid and that the destructive effect of stewing is small, and, for practical purposes, negligible.

Summary

1. Small samples of vegetables were immersed in boiling water for ½ hour, then analysed for α-tocopherol.

2. The average of the effects with 5 vegetables was a loss of only 3.7 % of the tocopherol.

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Original Communications

Alpha-Tocopherol Content of Foods

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THE acceptance of vitamin E as an essential nutrient in human nutrition and its relationship to polyunsaturated fatty acid intake in the diet has focused attention on the fact that the available data on the vitamin E content of foods are rather limited.

The most extensive series of analyses of various foods was carried out by Harris et al.[1] fourteen years ago. Improvements in analytical technics, particularly development of paper[2] and column chromatography[3, 4] and gas-liquid chromatography[5–7] offer the opportunity to re-evaluate the earlier data on the basis of technics known to be more accurate and able to differentiate between the α-tocopherol and the other, less active or completely inactive, forms of tocopherol.

Herting and Drury[8] using modern analytical technics reported the tocopherol values obtained in their laboratories over a four year period on a wide variety of vegetable oils. These investigators also summarized the available literature values. Other more recent publications report the α-tocopherol content of vegetables and fruits,[9, 10] margarines[11] and nuts.[12]

The need for new data on the α-tocopherol content of a wide variety of foods using modern analytical technics prompted the present investigation.

MATERIALS AND METHODS

Selection of Food Samples

The "typical" diet is unfortunately a matter of controversy and not fact so that any selection of foods is a compromise. In selecting the foods to be analyzed an attempt was made to get the most representative sampling possible within reasonable limitations. It is generally recognized that vegetable fats and oils are the principal source of vitamin E in the diet but since there are fairly ample data in the literature on the vitamin E content of vegetable oils *per se*, emphasis was placed on selecting foods containing or processed in vegetable oils or shortening. This seemed reasonable since a considerable proportion of the vegetable oil in the diet is consumed in this manner. Samplings of other foods represented a cross section of the American dietary including beverages, condiments and confections. Although many of the latter types of food were not expected to contain any significant quantity of vitamin E, they were included since many of them, e.g., coffee, are consumed in considerable quantities.

Source of Materials

All the food samples were purchased in local grocery stores. In some instances, several brands of one type of food were selected with particular em-

From Hoffmann-La Roche Inc., Nutley, New Jersey.
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phasis on those foods processed in vegetable oil and which, therefore, should be significant sources of vitamin E.

Preparation of Samples

Fresh foods were prepared and cooked, when applicable, in the laboratory test kitchen. Frozen foods were prepared according to the directions given on the package. Special emphasis was placed on this aspect of the study since it was considered important to assay the particular food in question just as it would be served and eaten.

As a check on the stability of tocopherol in foods deep fat fried in vegetable oils, batches of potato chips and french fried potatoes were prepared in the test kitchen by deep frying in fresh cottonseed oil. The frying oil was assayed before and after frying, and the fried foods were assayed fresh and after storage at room temperature and at −12°c.

Additional experiments were run to determine the relative stability of tocopherols and α-tocopheryl acetate in vegetable oils stored at high temperatures for prolonged periods. This was accomplished by heating various samples of vegetable oils with and without added *dl*-α-tocopheryl acetate in open beakers with an oil depth of 1 inch for 3 hours at 200 to 210°c. The α-tocopheryl acetate was added at a level of 100 mg. per 100 gm. of oil.

Method of Assay

The assay procedures used in these studies represent the contributions of many investigators but in general they follow the methods described in detail by Pudelkiewicz et al.,[13] Dicks et al.[14] and Herting and Drury.[8] The various steps include extraction, saponification without neutralization, re-extraction of the unsaponifiable material in Skellysolve B, chromatography on Florex XXS, separation of α-tocopherol by column chromatography on secondary magnesium phosphate and measurement of the tocopherol by the Emmerie-Engel method. Recoveries of α-tocopherol when carried through these analytical steps have been found to range between 95 to 100 per cent.

Solid foods were ground and homogeneously mixed in preparation for extraction. Fruits, vegetables, meats, bread, cake and other samples of this nature were homogenized by high speed blending for very short periods in a Lourdes Omni-Mixer. This type of blender is very efficient and can be kept chilled during operation; heat rarely develops during the operations. Manual chopping and blending were used when practical. Dry, friable materials, such as crackers, cookies, dry cereals and potato chips, were best prepared by crushing and kneading the sample in a small plastic bag. Cooked meats also yielded to cutting and chopping by hand. A very homogeneous sample could be obtained readily with relatively gentle handling.

When mechanical blending was employed, the sample was kept chilled, blending time was held to a minimum and the container and contents were flushed with nitrogen prior to blending to prevent any possible loss of tocopherols. Liquid and semi-solid samples (margarine, mayonnaise, ice cream and oils) required no special preparation and could be saponified directly.

The extraction and all subsequent steps in the assay are performed in subdued artificial light and preferably in amber apparatus. Two methods of extraction were employed in the assays: (1) For low moisture samples with an appreciable fat content, a Skellysolve B extraction in a Lourdes Omni-Mixer was the method of choice. Cookies, crackers and potato chips, which could be reduced easily to a fine powder, were extracted in this manner. (2) The second method was continuous extraction with anhydrous ethanol. This is performed either in a large Soxhlet unit or by a hot vapor system. The ethanol gives a complete and relatively clean extract with fruits, vegetables, meats, cereals and other products when tissue maceration and penetration are required for complete extraction of the tocopherols. The extraction time is 18 hours for this method.

The use of a further means of purification was required in several of the foods assayed. The failure of the Florex to remove the carotenoids and other pigments from tomato juice and carrots resulted in the need for an additional extraction of these products with a 60 per cent sulfuric acid solution. This was carried out after saponification with the purified extract in Skellysolve B. A Florex column was then used to obtain a clear, colorless solution.

One brand of ice cream assayed in the study required the use of a methanol "freeze-out" at −10°c. to remove unsaponified lipid-like material.[15] The failure of the magnesium phosphate column to absorb any of the tocopherols was remedied by the freeze-out.

RESULTS AND DISCUSSION

The results obtained on a wide variety of foods are listed in Table 1. They are subdivided into types of foods for convenience in comparison and reference use. The total tocopherol column actually represents total reducing substances as measured just prior to

TABLE I
Vitamin E Content of Foods

Product	Total Tocopherol (mg. %)	α-Tocopherol (mg. %)	% Lipid	α-Tocopherol (mg./gm. Lipid)
Meats				
Bacon (fried)	0.59	0.53	39.7	0.001
Ham steak (fried)	0.52	0.28	6.8	0.04
Pork sausage (fried)	0.32	0.16	35.0	0.005
Liverwurst	0.69	0.35	27.0	0.013
Bologna	0.49	0.06	26.6	0.002
Salami	0.68	0.11	16.0	0.007
Ground beef (pan fried)	0.63	0.37	11.1	0.033
Fresh beef liver (broiled)	1.62	0.63	5.8	0.11
Fresh veal cutlet (pan fried)	0.24	0.05	1.6	0.03
T bone beef steak (broiled)	0.55	0.13	9.5	0.014
Lamb chops (broiled)	0.32	0.16	13.3	0.012
Pork chops (pan fried)	0.60	0.16	19.5	0.008
Fish				
Fillet of haddock (broiled)	1.20	0.60	0.18	3.3
Salmon steak (broiled)	1.81	1.35	3.20	0.42
Deep fried frozen shrimp				
Oven heated	6.6	0.6	12.9	0.047
Not heated	5.9	1.9	11.5	0.165
Deep fried frozen scallops				
Oven heated	6.2	0.60	9.4	0.064
Not heated	3.9	0.71	5.9	0.12
Poultry				
Chicken breast (broiled)	0.58	0.37	3.90	0.095
Frozen fried chicken				
Brand "A"				
Oven heated	0.32	0.04	23.8	0.002
Brand "A-1"*				
Oven heated	1.39	0.38	20.0	0.019
Not heated	1.43	0.40	19.0	0.021
Brand "B"				
Oven heated	1.10	0.16	18.0	0.009
Not heated	0.80	0.10	14.0	0.007
Vegetables				
Raw potato	0.085	0.053	...	...
Baked potato	0.055	0.027	...	...
Boiled potato	0.061	0.043	...	...
Frozen french fried potatoes				
Brand "A"				
Oven heated	0.36	0.12	6.8	0.018
Not heated	0.64	0.15	5.5	0.027
Brand "B"				
Oven heated	1.59	0.43	6.2	0.069
Not heated	1.22	0.41	5.4	0.076
Fresh yellow onion	0.34	0.22	...	...
Frozen french fried onion rings				
Brand "A"				
Oven heated	6.2	0.72	25.6	0.028
Not heated	5.2	0.60	20.6	0.029

* Same as "A" but bought at a different store.

TABLE I (*continued*)
Vitamin E Content of Foods

Product	Total Tocopherol (mg. %)	α-Tocopherol (mg. %)	% Lipid	α-Tocopherol (mg./gm. Lipid)
Vegetables (continued)				
Brand "B"				
Oven heated	6.4	0.65	23.2	0.028
Not heated	5.5	0.52	17.2	0.030
Baked beans, Boston style	1.16	0.14	...	...
Fresh peas	1.73	0.55	1.74	0.32
Canned green peas	0.04	0.02	...	...
Frozen green peas				
Cooked	0.65	0.25	...	...
Uncooked	0.64	0.22	...	...
Canned green beans	0.05	0.03	...	...
Frozen cut green beans				
Cooked	0.25	0.11	...	...
Uncooked	0.24	0.09	...	...
Canned leaf spinach	0.06	0.02	...	...
Canned kernel corn	0.09	0.05	...	...
Frozen kernel corn				
Cooked	0.48	0.19	...	...
Uncooked	0.49	0.19	...	...
Celery	0.57	0.38	0.116	3.3
Carrots	0.21	0.11	0.046	2.4
Lettuce	0.17	0.061	0.112	0.55
Fresh tomatoes	0.85	0.40	0.074	5.4
Dry navy beans	1.68	0.47	0.60	0.78
Cooked white rice	0.27	0.18	0.50	0.36
Fruits and Fruit Juices				
Fresh strawberries	0.29	0.13	0.128	1.02
Frozen sliced strawberries	0.40	0.21	0.069	3.04
Fresh banana	0.42	0.22	0.29	0.76
Fresh cantaloupe melon	0.31	0.14	0.20	0.70
Fresh apple	0.51	0.31	0.19	1.63
Canned tomato juice	0.71	0.22	...	...
Canned grapefruit juice	0.18	0.04	...	...
Fresh orange juice	0.20	0.04	...	...
Breads				
White bread	0.23	0.10	2.6	0.05
Whole wheat bread	2.2	0.45	2.8	0.16
Cereals				
Oatmeal	3.23	2.27	6.33	0.36
Corn flakes	0.43	0.12	0.33	0.36
Dry processed rice cereal	0.28	0.04	0.2	0.2
Dry oat cereal	1.53	0.60	2.3	0.26
Yellow corn meal	3.43	0.64	3.80	0.17
Hominy grits	1.17	0.31	0.9	0.34
Processed wheat and barley cereal	2.45	0.61	0.98	0.62

TABLE I (*continued*)
Vitamin E Content of Foods

Product	Total Tocopherol (mg. %)	α-Tocopherol (mg. %)	% Lipid	α-Tocopherol (mg./gm. Lipid)
		Desserts		
Chocolate ice cream				
Brand "A"	1.02	0.36	12.2	0.03
Brand "B"	1.10	0.37	10.7	0.035
Vanilla ice cream	0.39	0.06	13.2	0.005
Fresh baked apple pie	15.7	2.50	10.6	0.24
Fresh baked blueberry pie	17.7	3.12	14.7	0.21
Fresh pound cake	7.4	1.1	22.0	0.05
Chocolate cupcake	2.0	0.14	17.2	0.008
Milk chocolate bar	4.2	1.1	29.8	0.037
Peanut butter/oatmeal cookie	7.67	6.0	24.0	0.25
Shortbread cookie	1.33	0.46	24.7	0.02
Wafer type cookie	1.43	0.53	10.8	0.05
Chocolate/cream cookie	2.81	1.29	17.5	0.07
		Miscellaneous		
Mustard	4.15	1.75	3.50	0.50
Whole milk	0.093	0.036	3.40	0.011
Butter	1.0	1.0	...	...
Egg	1.43	0.46	7.0	0.066
Corn oil margarine, brand "A"	46.7	13.2	80.0	0.17
Soya and cottonseed oils margarine, brand "B"	59.5	13.0	82.0	0.16
Cocktail peanuts	11.2	6.7	50.3	0.13
Dry roasted peanuts	11.7	7.7	49.3	0.15
Instant coffee	0.48	nil	...	...
Cottonseed oil mayonnaise, brand "A"	9.0	6.0	11.0	0.54
Mayonnaise, brand "B"	50.0	24.3	66.0	0.37
Polyunsaturated mayonnaise, brand "C"	42.0	8.6	76.4	0.11
Potato chips	11.4	6.4	44.8	0.14
Pretzel sticks	0.77	0.15	1.62	0.09
Club cracker	1.17	0.80	16.6	0.05

magnesium phosphate chromatography so that any artifacts which are not removed by the previous purification steps will be included. The α-tocopherol column, however, represents only α-tocopherol plus zeta$_1$- and zeta$_2$-tocopherol, if present. Also shown in the table are the lipid content and the α-tocopherol content expressed on a lipid basis.

As would be expected, the data reveal that the most important sources of vitamin E in the diet are vegetable oils and foods containing significant amounts of vegetable oils or shortening. The actual amount of α-tocopherol supplied by a vegetable oil-containing food, however, appears to be dependent on the processing and storage to which the food was subjected before eating. The processing losses which are actually measured in this study involve only the cooking of the food and not the losses involved in the commercial refining or preparation of the food for the consumer. In general, it can be concluded from this study that the ordinary cooking processes used in preparing food for the table do not involve very large losses of tocopherol. The most significant losses of tocopherol occur during the storage of foods which have been cooked in vegetable oils.

Today, practically all the commercial deep fat fried frozen foods, such as french fried potatoes, scallops, shrimp and chicken, are prepared in vegetable oils or shortening. Therefore, these

TABLE II
Stability of Tocopherol in Fried Foods

Data	Total Tocopherol (mg. %)	% Loss
Tocopherol content of oil		
Before frying	82	...
After frying	73	11
Tocopherol content of oil extracted from potato chips		
Initial (after preparation)	75	...
2 wk. at room temperature	39	48
1 mo. at room temperature	22	71
2 mo. at room temperature	17	77
1 mo. at −12°c.	28	63
2 mo. at −12°c.	24	68
Tocopherol content of oil extracted from french fried potatoes		
Initial	78	...
1 mo. at −12°c.	25	68
2 mo. at −12°c.	20	74

foods should supply a significant amount of α-tocopherol in the diet. However, examination of the data reveals that this is not so. The relatively low tocopherol content in a wide variety of such frozen foods prompted the investigation of the stability of tocopherol in foods fried in vegetable oil and placed in freezer storage (Table II). While there is little loss of tocopherol during deep fat frying in fresh vegetable oil, there is a great loss of tocopherol in potato chips stored at room temperature and in french fried potatoes and potato chips stored at −12°c.

This loss of tocopherol in frozen foods is of considerable importance because of the relatively large consumption of frozen foods in the United States today. The reason that low storage temperatures do not prevent the oxidation of tocopherols is probably related to the fact that the formation of hydroperoxides is not prevented by low temperatures. It is generally accepted that hydroperoxides are one of the first degradation steps in the oxidation of unsaturated fatty acids.[16] These hydroperoxides, however, are relatively unstable, and at slightly elevated temperatures they change to peroxides which in turn further decompose by cleavage to give aldehydes and ketones. It is these latter compounds which give the organoleptic evidence of rancidity. At freezer temperatures this stepwise oxidation proceeds at much slower rates so that appreciable quantities of the relatively unstable hydroperoxides accumulate. Since hydroperoxides readily destroy tocopherol, this accounts for the large losses of tocopherol which occur during freezer storage despite good commercial packaging practices.

If the vitamin E nutritional status of fried frozen foods is to be maintained, it appears, that the easiest method of doing so is to fortify the vegetable oils used in the preparation of these foods with α-tocopheryl acetate- a form of vitamin E which is stable to heat and oxidation. Table III shows the relative stability of tocopherols and *dl*-α-tocopheryl acetate in vegetable oils exposed to high temperatures. While losses of unesterified tocopherols found in this test may be greater than would be found in deep fat frying with less surface to volume ratio, it does serve to illustrate the good stability of the ester form of α-tocopherol to heat and oxidation.

Referring again to Table I, it will be noted that fruits and vegetables do not contribute significant amounts of α-tocopherol to the diet. The tocopherol content of canned vegetables is low in comparison to fresh or frozen vegetables, e.g. fresh, frozen and canned peas contain 0.55, 0.25 and 0.02 mg. per cent α-tocopherol, respectively. Home cooking of vegetables, however, has a small destructive effect on tocopherol. This observation was also reported by Booth and Bradford[10] who found an average loss of 3.7 per cent tocopherol for five different vegetables cooked in boiling water for a half hour. Commercial processing and storage, however, apparently result in a considerable loss of tocopherol.

Meats, fish and poultry supply low to moderate amounts of α-tocopherol. Fish appears to be the best source of tocopherol in this group, with salmon steak having the highest α-tocopherol content of the samples assayed in this study.

Desserts can vary considerably in α-tocopherol content. Cookies and pies made with vegetable oil shortening can be very good

TABLE III

Stability of Tocopherol and Tocopheryl Acetate in Cooking Oils*

Oil	Initial Tocopherol Content (mg. %)	Per cent Tocopherol Destroyed	Per cent Tocopheryl Acetate Destroyed
Deodorized—unhydrogenated corn oil	100	97	13
Deodorized—hydrogenated corn oil	105	99	19
Deodorized—unhydrogenated cottonseed oil	91	97	19
Deodorized—hydrogenated cottonseed oil	80	99	8
Deodorized—unhydrogenated soybean shortening oil	101	96	12
Deodorized—hydrogenated soybean shortening oil	73	99	13
Stabilized safflower oil	59	97	6
Unstabilized safflower oil	36	98	5

* 100 mg. per cent *dl*-α-tocopheryl acetate added to each cooking oil.

sources of α-tocopherol. Snack foods, such as fresh potato chips cooked in fresh vegetable oil, can also be rich in α-tocopherol.

The α-tocopherol content of the foods shown in Table I was used to estimate the α-tocopherol content of a variety of menus. Estimates for the average portions in the menus were taken from Bowes and Church.[17] The α-tocopherol contents of eight typical breakfast, luncheon and dinner menus were calculated.* In selecting the menus a range of typical caloric intakes were chosen. The α-tocopherol content of the breakfast, luncheon and dinner menus ranged from 0.59 to 3.68, 0.44 to 5.37 and 1.61 to 6.38 mg. of α-tocopherol, respectively. Based on these data the daily intake of α-tocopherol would range, therefore, between 2.6 mg. to 15.4 mg. with an over-all average of 7.4 mg.

Harris and Embree,[18] using primarily the analytical data of Harris, Quaife and Swanson,[1] calculated the α-tocopherol content of various food groups available for consumption in the United States in 1960 and thereby estimated the average daily intake of α-tocopherol to be 11.9 mg. Although admittedly the latter value was derived on a broader general base, it is about double the value calculated by us from typical menus. This difference is probably partially due to the fact that α-tocopherol assays of foods *as eaten*, using modern analytical technics, tend to give lower values for many foods than are obtained on fresh, uncooked food. Also, in many cases, α-tocopherol values obtained by modern analytical technics differ from those obtained by older methods. These differences were pointed out in canned and frozen vegetables and in deep fat fried frozen foods. While the average daily α-tocopherol intake of 7.4 mg. calculated from typical menus is reasonably accurate for these particular menus, it is not intended to imply that this value actually represents the so-called "average" daily intake in the United States. It does indicate, however, that a variety of meals actually supply daily intakes of α-tocopherol considerably below 15 mg. In order to reach a daily intake of α-tocopherol of this magnitude, it was necessary to add up the tocopherol contents of the breakfast, luncheon and dinner menus which were highest in tocopherol content. It therefore appears that it would be necessary to provide a relatively good source of fresh vegetable oil or its equivalent in the diet every day to maintain a daily intake of α-tocopherol of approximately 15 mg.

The dependency of the requirement for vitamin E on the intake of polyunsaturated fatty acids has been well established by many investigators in both man and animals and well summarized in the paper by Harris and Embree.[18] The pattern of the consumption of various vegetable oils is, therefore, of foremost

* Copies of these menus may be obtained by writing to us.

TABLE IV
U. S. Consumption* of Various Vegetable Oils

Year	Margarine	Salad Dressing	Shortening	Total	Pounds per Capita
			Soybean Oil		
1946	201	70.7	744	1,015.7	7.3
1955	746	312.3	930	1,988.3	12.3
1963	1,058	527.3	1,201	2,796.3	14.9
			Cottonseed Oil		
1946	254	93.0	502	849.0	6.2
1955	278	127.6	439	844.6	5.2
1963	100	156.5	330	586.5	3.2
			Peanut Oil		
1946	10	0.1	42	52.1	0.4
1955	2	...	6	8.0	0.05
1963	4	...	3	7.0	0.03
			Corn Oil		
1946	9	56.4	3	68.4	0.5
1955	0.5	29.4	3	32.9	0.2
1963	113	22.6	4	139.6	0.8
			Total Oil Consumption		
1946	474	220.2	1,291	1,985.2	14.4
1955	1,026.5	469.3	1,378	2,873.8	17.7
1963	1,275	706.4	1,538	3,529.4	19.0

* Amounts used in million pounds. Total population: 1946, 138.4 million; 1955, 162.3 million; 1963, 186.6 million.

importance in arriving at an estimate of the adequate dietary intake of vitamin E. This approach was used by the latter investigators in estimating the adequacy of the vitamin E content of the American diet. Of particular interest to us is the gradual change in the consumption of polyunsaturated fatty acids which has occurred over the past years. For this comparison the years 1946, 1955 and 1963 were chosen and the data were taken from the March and August 1964 Fats and Oils Situation Reports of the Economic Research Service of the U. S. Department of Agriculture. Table IV shows the consumption of the principal vegetable oils and Table V the consumption of butter and lard. It can be seen that there is a general trend toward increased consumption of vegetable oils and decreased consumption of animal fats. Particularly marked is the large increase in the use of soybean oil. Since this oil only averages about 15 mg. per cent α-tocopherol it does not supply a correspondingly large increase

TABLE V
U. S. Consumption* of Animal Fats

Year	Butter	Lard
1946	10.5	11.8
1955	9.0	10.1
1963	6.8	6.5

* Pounds per capita.

in α-tocopherol intake as would have been the case with cottonseed oil which averages about 35 mg. per cent α-tocopherol. A resulting imbalance in the vitamin E:polyunsaturated fatty acid (E:PUFA) ratio is therefore possible in many foods which are based on soybean oil which has not been hydrogenated.

Obviously more data are needed, using modern analytical technics, to obtain a clearer picture of the actual vitamin E content of the so-called "average" diet. The ideal situation would be to perform both α-tocopherol and polyunsaturated fatty acids assays on the same food sample. Data of this type would give a much truer picture of the actual E:PUFA ratio of the food which is being consumed and are really necessary before a decision can be made on whether the vitamin E content of a diet is adequate or inadequate. The technic used by Harris and Embree[18] in estimating the α-tocopherol and polyunsaturated fatty acid content of the average diet represents *disappearance* of foods rather than the amount *actually eaten*. This does not take into account wastage, which in the case of cooking and salad oils could be considerable, or the losses incurred in the processing, cooking and storage of foods. The α-tocopherol content of the foods which were assayed and compiled into menus in this study indicates the possibility of relatively low α-tocopherol intake in a portion of the population, depending somewhat on dietary habits. This observation, therefore, points up the importance of obtaining more data to more accurately estimate the adequacy of vitamin E in the American diet.

SUMMARY

A variety of foods were assayed for α-tocopherol content using modern analytical technics. An important feature of this study was to assay foods *as eaten* to obtain a more accurate picture of the actual α-tocopherol intake in the diet. Of particular interest was the finding that frozen foods which were fried in vegetable oil were surprisingly low in tocopherol indicating severe losses of tocopherol during freezer storage.

The α-tocopherol content of typical breakfast, luncheon and dinner menus was calculated using the values obtained in this study. These menus indicated a daily intake of α-tocopherol ranging from 2.6 to 15.4 mg. with an over-all average of 7.4 mg. This value is about half of previous estimates of daily vitamin E intake and therefore indicates the possibility of relatively low α-tocopherol intake in a portion of the population, depending somewhat on dietary habits.

This observation points up the importance of doing more work in this area to establish more accurately the actual intake of both vitamin E and polyunsaturated fatty acids in order better to assess the adequacy of vitamin E in the "average" American diet.

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THE EFFECT OF VITAMINS B_{12}, E, CHOLINE, AND THEIR COMBINATIONS ON THE STATE OF THE ANTICOAGULATIVE SYSTEM IN CONDITIONS OF EXPERIMENTAL HYPERCHOLESTEROLEMIA

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Vitamins B_{12}, E, and choline are finding use in the treatment of disorders accompanied by an increase in the coagulative capacity of the blood - during atherosclerosis and stenocardia [1-3, 6]. The effect of these vitamins on the anticoagulative system, and (on) depression, which may have to do with the increased coagulability of blood during atherosclerosis [4, 5], is not known. Only isolated works are available concerning the effect of vitamin E on the separate humoral agents of the anticoagulative system. Thus, Bottiglioni and Facchini [8] traced the activation of heparin in a majority of patients studied after introducing them to vitamin E in medicinal dosages. Santangelo [10] noted a depression of fibrinolysis in children receiving vitamin E over the course of 2 days. Tomoda Yutaka [11] observed the opposite effect in experimentation - activation of fibrinolysis among rabbits into which vitamin E was introduced.

We did not encounter data concerning the effect of vitamin B_{12} and choline on any humoral agents of the anticoagulative system.

In current work, we studied the effect of vitamins B_{12}, E, and choline on the state of the following anticoagulative systems indicators: 1) fibrinolytic activity (euglobulin time according to Kowalski, Konec, (and) Niewiarowski [9]) and fibrin loss in the incubative coagulum according to Bidwell; 2) the amount of fibrinogen according to Bidwell; 3) free heparin level according to E. Sirman [7]; 4) antithrombin activity as expressed in percent (by) the relation-

ship of thrombin plasma time in experimental and intact animals. In tests concerning alimentary hypercholesterolemia, the amount of cholesterol in the blood was determined according to Engel'gardt - Smirnova.

Blood specimens for all determinations were taken from the jugular vein. Tests (were) conducted on male rats weighing from 150 to 350 g. All test animals received a standard casein-starch ration, containing a salt mixture and a collection of vitamins in accordance with recommendations of the Institute of Nutrition of the Soviet Academy of Medical Sciences. The investigated vitamins were supplementarily introduced into the composition of the ration in the following amounts: vitamin B_{12} - 20 mkg. (micrograms), vitamin E - 0.15 mg., and choline - 25 mg. per animal per day. In tests with combined charges, vitamins were introduced in these same dosages. Animals of the controlled group received a rich ration, containing vitamins in accordance with the daily requirement for rats.

In preliminary tests, indicators of the state of the anticoagulative system during the excessive introduction of vitamin B_{12}, choline, and vitamin E over the course of 10 days were studied (table 1).

Table 1

Indicators of the state of the anticoagulative system in animals receiving an excess of vitamins B12, E, and choline over the course of 10 days

TEST CONDITIONS	NUMBER OF RATS	FIBRINOLYTIC ACTIVITY		AMOUNT OF FIBRINOGEN (IN %)	FREE HEPARIN (IN SEC.)	[illegible] ACTIVITY (IN %)
		[illegible] (IN HOURS)	[illegible] (IN %)			
[illegible]	8	1,8±0,10	24,0±1,0	0,22±0,008	8,8±0,6	99±2,2
EXCESS OF VITAMIN B_{12}	6	2,8±0,10 (100,0%)	14,0±1,1 (100,0%)	0,26±0,010 (99,1%)	8,1±0,6 (56,3%)	66±2,2 (100,0%)
EXCESS OF VITAMIN E	7	1,7±0,09 (56,2%)	25,0±1,1 (56,2%)	0,23±0,010 (55,8%)	9,0±1,0) (15,5%)	94±2,5 (84,2%)
EXCESS OF CHOLINE	6	3,2±0,13 (100,0%)	13,0±0,9 (100,0%)	0,21±0,009 (86,4%)	8,0±0,8 (55,9%)	6[illegible]±1,7 (100,0%)

Annotation (to Table 1). 1. Here and in the following tables, average amounts and the average inaccuracy are presented. 2. The values of P, calculated in relation to the controlled group, are presented in parentheses.

From table 1 it is apparent that a supplementary lot of vitamin B_{12} caused a prolongation of euglobulin time and a decrease of fibrin loss, i.e., a depression of fibrinolysis. Moreover, the fibrinogen level was increased somewhat and antithrombin activity was decreased. An excess of choline caused similar changes, but with the difference that in this instance, a fibrinogen increase is not noted. The supplementary introduction of vitamin E was not reflected in the amount of investigated indicators.

In the second series of investigations, the effect of vitamins B_{12}, E, and choline on the state of the anticoagulative system in conditions of alimentary hypercholesterolemia is studied. Animals in this series of tests received a rate of 3 mg. of 6-methylthiouracil daily over the course of the first 30 days and a rate of 0.2 g. of cholesterol over the extent of the entire test, continued (for) 90 days. The day before the test, (and) on the 30th, 60th, and 90th day, blood samples were taken. Results of the study of humoral agents of the anticoagulative system before the start of testing and on the 90th day of testing are presented in table 2.

From the data presented in table 2, it is apparent that, till the end of testing, in line with the development of cholesterolemia in animals of all groups, a depression of fibrinolytic activity (a prolongation of euglobulin time and a decrease of fibrin loss in the incubative coagulum), a decrease of free heparin level, a depression of antithrombin activity and an increase of fibrinogenemia are observed. The combination of these changes bears on the depression of the anticoagulative system. The extent of the described changes was found to be different in the various tests groups. Thus,

Table 2

Indicators of the state of the anticoagulative system and the amount of cholesterol in the blood among rats receiving a vitamin excess in a standard ration base containing 6-methylthiouracil and cholesterol

Test conditions	Number of rats	Sample extraction time	Fibrinolytic activity: Euglobulin time (in hours)	Fibrinolytic activity: Fibrin loss (in %)	Amount of fibrinogen (in %)	Free heparin (in sec.)	Antithrombin activity (in %)	Amount of cholesterol (in mg. %)
Control	12	Day before testing	1,8±0,03	23,4±1,2	0,22±0,007	8,3±0,5	98±2,3	62±2,3
		On the 90th day of testing	3,3±0,11 (100,0%)	17,0±1,1 (100,0%)	0,32±0,011 (100,0%)	3,3±0,3 (100,0%)	70±1,7 (98,9%)	287±3,7 (100,0%)
Excess of vitamin B_{12}	12	Day before testing	1,7±0,07	23,0±0,9	0,22±0,009	8,0±0,4	96±2,2	65±2,0
		On the 90th day of testing	3,8±0,06 (99,7%)	12,0±1,0 (99,9%)	0,32±0,010 (100,0%)	2,3±0,4 (90,4%)	69±2,6 (97,6%)	269±3,9 (99,9%)
Excess of vitamin E	12	Day before testing	1,7±0,06	23,0±0,8	0,22±0,003	8,0±0,4	96±1,6	63±2,4
		On the 90th day of testing	4,2±0,10 (100,0%)	10,0±1,1 (100,0%)	0,33±0,009 (51,6%)	1,7±1,9 (100,0%)	68±1,9 (99,9%)	251±4,1 (87,6%)
Excess of choline	12	Day before testing	1,8±0,06	25,0±1,0	0,25±0,009	8,0±0,4	96±2,4	67±2,8
		On the 90th day of testing	3,7±0,11 (99,1%)	14,0±1,0 (96,4%)	0,34±0,009 (83,9%)	2,5±0,4 (89,0%)	65±2,6 (99,9%)	264±3,2 (99,9%)

Annotation. The values of P (presented in parentheses) are calculated in the control group by relationship to size determined before the start of testing; in the remaining groups - by relationship to the size found in control animals on the 90th day of testing.

during the excessive introduction of vitamin B_{12}, the depression of fibrinolysis, the decrease of free heparin level, and the depression of antithrombin activity is more marked than in the control animals. During the excessive introduction of choline, the extent of changes in the investigated indicators is approximately the same as during the excessive introduction of vitamin B_{12}.

Changes produced by an excess of vitamin E proved to be somewhat surprising. As is known from preliminary tests, vitamin E did not cause a depression of the anticoagulative system during a 10-day introduction in a full-value ration base. In this ration base, an excess of vitamin E caused a depression of the anticoagulative system, expressed to a greater degree than during an excess of vitamin B_{12} or choline, leading to the development of hypercholesterolemia. A significant decrease of fibrinolytic activity and free heparin level in animals receiving a vitamin E excess attests to this.

In the third series of investigations, changes in humoral agents of the anticoagulative system during the excessive introduction of vitamins B_{12}, E, and choline are studied in the following combinations: 1) vitamin B_{12} and choline; 2) vitamin E and choline; 3) vitamins B_{12} and E, and; 4) vitamins B_{12}, E, and choline. The enumerated vitamin combinations were introduced, as in preceding tests, in a ration base, leading to the development of hypercholesterolemia. Results of the study of humoral agents in the anticoagulative system the day before testing and on the 90th day of testing are presented in table 3.

As seen in table 3, changes, already known from the preceding investigations, took place in animals of the control group till the termination of testing. In animals receiving an excess of vitamin B_{12} and choline, a depression of the anticoagulative system is expressed to the same extent as observed during the separate introduction of these vitamins on a background of hypercholesterolemia. During the combined

Table 3

Indicators of the state of the anticoagulative system and amount of cholesterol in the blood in rats receiving an excess of vitamins in various combinations in a standard ration base containing 6-methylthiouracil and cholesterol

Test conditions	Number of rats	Sample extraction time	Fibrinolytic activity: Euglobulin time (in hours)	Fibrinolytic activity: Fibrin loss (in %)	Amount of fibrinogen (in %)	Free heparin (in sec.)	Antithrombin activity (in %)	Amount of cholesterol (in mg. %)
Control	10	Day before testing	2.3±0.08	21.0±0.8	0.25±0.0010	6.7±0.7	90±1.4	74±3.0
		On the 90th day of testing	3.9±0.13 (100.0%)	15.0±0.9 (100.0%)	0.38±0.012 (100.0%)	3.5±0.5 (100.0%)	66±1.8 (100.0%)	305±1.8 (100.0%)
Excess of vitamin B_{12} and choline	10	Day before testing	2.2±0.09	24.0±1.0	0.26±0.12	7.2±0.9	97±2.0	76±4.3
		On the 90th day of testing	4.8±0.11 (100.0%)	13.0±0.9 (91.1%)	0.37±0.013 (45.2%)	1.6±0.2 (100.0%)	58±1.2 (99.9%)	225±8.0 (100.0%)
Excess of vitamin E and choline	10	Day before testing	2.1±0.01	24.0±0.8	0.25±0.010	7.3±0.6	94±2.6	72±3.7
		On the 90th day of testing	5.2±0.10 (100.0%)	11.0±0.7 (100.0%)	0.36±0.009 (80.6%)	1.4±0.12 (100.0%)	61±2.6 (97.9%)	310±7.0 (45.2%)
Excess of vitamins B_{12} and E	10	Day before testing	2.1±0.12	22.0±1.1	0.23±0.012	7.7±0.8	90±2.1	69±3.5
		On the 90th day of testing	5.5±0.15 (100.0%)	9.0±0.9 (100.0%)	0.40±0.014 (72.9%)	1.5±0.16 (100.0%)	55±1.4 (100.0%)	313±10.1 (80.6%)
Excess of vitamins B_{12}, E, and choline	10	Day before testing	2.0±0.09	25.0±0.8	0.23±0.009	8.5±0.7	94±1.2	73±3.0
		On the 90th day of testing	5.6±0.14 (100.0%)	9.0±0.97 (100.0%)	0.42±0.011 (99.1%)	1.9±0.2 (100.0%)	56±1.5 (100.0%)	303±10.3 (15.8%)

Annotation. The values of P were calculated in the control group by relationship to size, determined before testing; in the remaining groups - by relationship to the size found in control animals on the 90th day of testing.

introduction of an excess of vitamin E and choline, the extent of changes in the anticoagulative system does not exceed that (observed) during the introduction of only vitamin E. The joint introduction, in excess, of vitamins B_{12} and E caused a change in the anticoagulative system exceeding that which was observed during the separate introduction of these vitamins. The combined introduction of an excess of vitamins B_{12}, E, and choline does not effect the degree of changes in the anticoagulative system produced by the combination of vitamins B_{12} and E.

Consequently, only during the combination of an excess of vitamins B_{12} and E in a ration base, leading to the development of hypercholesterolemia, is a partial summation of the depression effect in the anticoagulative system observed. The excessive introduction of vitamins in other combinations caused a depression of the anticoagulative system, but the degree of expression of a corresponding maximum effect is produced by one of the components of the combinations. Thus, the supplementary introduction into white rats of an excess of vitamin B_{12} or choline in a standard full-value ration over the course of 10 days, produces a depression of fibrinolysis and antithrombin activity. The introduction of an excess of vitamin E in these same conditions does not effect corresponding indicators.

The excessive introduction of vitamin B_{12} or choline in a ration, leading to the development of hypercholesterolemia, worsens depression of the anticoagulative system, accompanied by hypercholesterolemia.

An excess of vitamin E in the composition of a ration, leading to the development of hypercholesterolemia, worsens depression of the anticoagulative system to a greater degree than an excess of vitamin B_{12} or choline.

During the combined introduction of an excess of vitamins B_{12} and E on a background of hypercholesterolemia, their effect on the anticoagulative system is partially summarized.

During the introduction of other combinations, a summation is not observed.

The depressing effect of the investigated vitamins on the anticoagulative system can not be related to their effect on the level of cholesterolemia, inasmuch as the level of cholesterol in the blood of all tested groups differed from (that) of the control (group) to an insignificant degree.

In the treatment of conditions distinguished by increased coagulability of the blood, the use of these vitamins desirably entails control of the anticoagulative system, particularly, of fibrinolysis.

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THE EFFECT PRODUCED BY VITAMINS B_{12}, E, CHOLINE AND BY THEIR COMBINATIONS ON THE STATE OF THE ANTICOAGULATIVE SYSTEM IN EXPERIMENTAL HYPERCHOLESTERINAEMIA

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Summary

Experiments conducted on albino rats ascertained that an excess administration of vitamins B_{12} and choline tends to depress fibrinolysis and antithrombin activity. In analogous conditions vitamin E excess does not affect the anticoagulative system. Incorporation in the diet of excess vitamins B_{12}, E and choline, leading to the development of hypercholisterinaemia, tends to aggravate still more the inhibition of the anticoagulative system, observed under hypercholisterinaemia. This inhibiton becomes most marked with the vitamin E excess and following combined administration of vitamins B_{12} and E.

The use of the studied vitamins in the treatment of conditions involving an increased blood coagulability is recommended to practice under supervision over the anticoagulative system, and ,in particular, over fibrinolytic activtiy.

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THE EFFECTS OF SMALL DOSES OF VITAMIN E ON THE NERVOUS SYSTEM

by Paul Chauchard

(paper presented at the meeting of March 3 1942)

The precise qualitative evaluation of the functional changes in the nervous systems allows us to determine an excitant or depressant pharmacological activity of the principal vitamins on the nerve centers , not including states of avitaminosis, for small doses close to the minimum daily requirement for the organism. The proper functioning of the nerve cells requires an optimum proportion of these vitamins. The water-soluble vitamins act on the encephalon and the medulla. There exists the possibility that these vitamins act beyond the conditions of avitaminosis. Some phenomena of synergy or of antagonism between the various vitamins are also possible, either in a normal state or in the various avitaminoses.

"The understanding of the role of vitamins in the maintenance of life and the normal functioning of the body is of primary importance". (Mrs. Randoin and Simmonet). However, if we begin to understand adequately the disturbances caused by the lack of various vitamins, to grasp the role they play in cell metabolism or in the regulation of endocrine activity, it is necessary to clarify the pharmacological or physiological properties of these substances. In general, "when vitamins are present, everything occurs normally". Also if the vitamins are considered so-called "anti substances" then one would be able to denote them by a positive action." Now that the principal vitamins, obtained in a pure state, in the form of definite chemical compounds , lend themselves easily to experimentation, the study of their action on the organism must be undertaken in the same manner as is done with all other pharmacodynamic substances.

Having available a refined and accurate study of the nerve functions, we propose to study the pharmacological activity of vitamins in this field, outside of any state of deficiency.

Similarly in neurology, vitamins are characterized primarily by the resultant effects of their deficient contribution, the nervous symptoms of avitaminosis. The vitamins themselves appear to have no action, at least in small doses, and they appear if we eliminate the possibility of side effects of a glycemian or calcemian type. In general, we pointed out only the nerve phenomena related to highly toxic doses, in cases of hypervitaminosis. Also vitamins have been conventionally considered as having only a weak pharmacological power. Let us point out, however, the existence of the vagomimetic properties for aneurin and ascorbic acid. Above all, let us point out

the curious crises resembling the polyneuritic irregularities obtained by Mrs. Rampoin and Lecoq in hhe case of a slight excess in vitamin B_1 in diets containing certain sugars.

The starting point of our study was to determine, during a study on B_1 avitaminosis of Mrs' Mazoue's rat, that the injection of aneurin caused changes in the excitability of the subjects, animals receiveing a sufficient daily quantity of aneurin. We have studied systematically the effects of the principal vitamins (A, B, PP, C, D, E) on the nervous excitability of the animals (rats and guinea pigs above all) who received normal nourishment and were not deficient. These substances were administered by intraperitoneal injections, after dissolution in a suitable solvent whose effectiveness we have verified. The simultaneous measurement of the changes in the peripheral motor chronaxies (extension and flexion of the toes), of a sensitive chronaxia and of a cortical motor chronaxia was carried out according to our usual method. The determinations were taken from the skin without the animal having received any hypnotic or anesthetic, while maintaining its normal behavior. The determinism of the observed changes was clarified by the research on the effects of general anesthesia (ether) or of a section of the nerve on ehe development of the phenomena. This permits revealing the actual location of the processes of nerve excitation or inhibition (the eneephalon, medulla or peripheral nerve).

In all cases we observed significant changes in excitability, indications of remarkable functional disorders of nerve functioning. Thus our study permits us to contemplate these vitamins as pharmacological agents, stimulants or depressants of the nervous system and to place them among these agents. Furthermore, these effects are obtained not with large doses such as with those which set into motion the irregularities of hypervitaminosis, but immediately after the injection of an extremely small dose, comparable to the daily dose necessary for maintaining the animal free from any deficiency. Thus this dose was sometimes smaller than those used for producing the same phenomena with substances such as gardenal or strychnine. One already notes a significant action with some doses on the order of one microgram (vitamins B_1 and A0 or greater than one milligram.

The variations in chronaxia indicate an excitant activity for A, C and D, and an inhibiting activity for PP. It is also diphasic, at first stimulating and then inhibiting for B_1 and E. Genrally speaking, with the exception of E, there is a tendency towards the equalization of the chroniaxias of the antagonists. The extent and length of the variations depend greatly on the injected dose of vitamins C, D, and, above all, B_1. Perhaps we might have the possibility here of a practical application for the dosage in certain states of concentration. The effect of the dose is almost negligible with vitamin A and weak with vitamin E.

All of the water-soluble vitamins which were studied (B_1, C, PP) have a purely encephalic action, whereas the liposoluble vitamins (A,D, and E) act further and in the same sense on the medulla. This extension of the focus of action of nervous activity must be due to the fact that liposoluble vitamins, because of their affinity to the nerve lipoids, adhere to the centers in a much more intense manner than water-soluble vitamins.

We have verified that carotene, provitamin A, has an activity which is rigorously opposed to that of this vitamin. It is an inhibitor of the encephalon and medulla. Ergesteron, provitamin D, stimulates the medulla, as does this vitamin, but , on the contrary, it inhibits the brain.

Given the smallness of the doses and the rapidity of the action, it must create a direct effect by the vitamins on the nerve cells, particularly as regards their metabolic functions, and not a secondary glycemic or endocrine effect. In the case of vitamin D, under 10,000 units, the activity noted is not related to hypercalcemia because otherwise this would completely modify the excitability, but at larger doses, this primary pharmacological effect is followed by a second phase of encephalic and medullar inhibition of hypercalcemic origin comparable to those described by Nitzesco, Angelasco and Timus in hypervitaminosis D.

In contrast to what occurs in the normal nerve stimulants or depressants, these processes of excitation or inhibition remain latent. They are revealed only by chronaxic changes, or sometimes by slight clinical signs (motor incoordination, excitation or depression of the animal) which has caused them to be unnoticed up to now. In the frog, however, we have been able to observe some true strycnine crises after the injection of several micrograms of aneurin. It is probable that certain of the nervous signs of hypervitaminosis are related to these pharmacological properties, particularly the seizures caused by a slight excess in vitamin B_1 (Randoin and Lecoq) and the convulsions of hypervitaminosis A.

Our experiments enable us to consider the possibility of synergic or antagonistic activities between the vitamins, based on their pharmacological power. They show that if the vitamins can act in the organism in a specific manner, as substances which the organism needs greatly, they can also have nonspecific effects. They may act as ordinary nerve stimulants or depressants, but are particularly favorable for the normal constituents of a non-toxified organism. We must thus be careful not to qualify the state of severe avitaminosis as including all of the states in which vitamins exhibit their activity, since such an activity is not necessarily a sign of avitaminosis, in contrast to the opinion of Szent-Gyorghi who did not allow for the possibility of a chemotherapeutic action of vitamins.

The functioning of the nervous system, and without a doubt this would be equally as true in other regions, thus requires an optimum proportion of vitamins in the same sense that it requires an optimum proportion of oxggen (Paul Bert). An excess and deficiency are also factors of disorders. Whereas the activity of deficiency is easy to perceive, the action of an excess is more difficult to bring into view. Under normal conditions of life support a physiological regulation of vitamins must come into play comparable to the liver regulation of vitamin A (Chevallier) without damage to its own cell regulation (potential action?).

A certain resemblance often exists between the effect of deficiency and pharmacological action: the location is the same, encephalic for the changes in excitability of avitaminosis B_1 and in the action of aneurin; encephalic and medullar for avitaminosis A and vitamin A, etc. Sometimes the activity is even direction: a decrease in the chronaxias by vitamin A as with avitaminosis A (Chevallier); excitant effects of small doses of vitamin B_1 throughout the duration of its condition. A parallel situation has been noted in the relationship between certain irregularities of avitaminosis and of hypervitaminosis (the cases of B_1 and A). This is found again in anoxia and hyperoxia.

In order to grasp in the most complete manner this problem on the activity of vitamins on the nerve centers, we must consider the possibility of the changes in sensitivity in the centers of the pathological conditions. We have been able to show as evidence, along with Mrs. Mazoue, such changes in the states of avitaminosis. In avitaminosis B_1 the curative action of aneurin, the temporary return to normal of the chroniaxias, after aneurin injection, results in a tranquillizing effect of this substance on the sensitivity of the centers, since this calming action normally only occurs in much larger doses of aneurin. The smaller doses begin by increasing the excitation of the deficient animal. The disorders of avitaminosis A, which, normally a stimulant, works as an inhibitor on the centers stimulated by the deficiency. Otherwise such effects can be obtained outside the realm of all specificity. A temporary correction of the disorders of excitability in avitaminosis A is obtained by injecting aneurin and of those in avitamosis B_1 by injecting ascorbic acid. These substances, which are normally stimulants, now become inhibitors. These curious phenomena relate on the one hand to the synergy which exists between the vitamins from the pharmacological point of view and, on the other hand, to the presence of a possible common factor such as acidosis (Lecoq) in different instances of avitaminosis.

Concerning nerve functioning we can see that our research leads us to new considerations which contributing the clarifying the physiological and pharmacological determinism (causation) of these vitamin activities which have not yet lost their complete mystery.

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ON THE POSSIBLE EFFECT OF VITAMIN E ON THE STRUCTURE AND FUNCTION OF THE THYROID

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In the sphere of a study of the correlations between vitamin E and the other vitamins, the hormones, and the enzymatic systems, the relationship between vitamin E and the thyroid incretion seems to assume particular interest because of the fact that, though the ultimate point of attack in the organism and the precise biological significance of both substances is unknown, the two are considered almost antagonistic in some metabolic moments, since thyroxine is a substance which favors oxidation, while vitamin E is a substance which might have the capability and function of inhibiting it.

This does not exhaust the range of biological activity of either of the substances; both are important for morphogenesis and the development of the body in general (thyroxine) or for some of its parts (vitamin E).

In order to document the possible correlations between vitamin E and the thyroid function, we examined:

I. The concentration of vitamin E in the blood: a) of normal individuals (before and after administration of varied doses of the vitamin); b) of hyperthyroidal men; c) of hypothyroidal patients.

II. The histological and functional modifications induced in the thyroid of an animal: a) upon administration of high, protracted doses of vitamin E; b) by subjecting the animal to a diet deprived of vitamin E.

III. The modifications induced by the administration of vitamin E (in quantities exceeding the norm) on the proper function of the thyroid and in those metabolic balances which appear to be most strictly governed by the thyroid, i.e.: a) the thyroid accumulation of iodine; b) the concentration of organic iodine in the serum; c) the basal consumption of oxygen; d) cholesterolemia e) the alkaline reserve; f) oxidated and reduced glutathionemia. This was tested in man and also, in part, in the animal.

We will cite the data of the bibliography with which we became acquainted in the course of the exposition of the data of our observation.

The following was determined by the micro-method of

1A. Costa, P. Monteferrario, O. Volterrani: Mauritian Hospital, Torino. G. Cetini: Institute of Physical Chemistry of the University, Torino.

Quaife et al. (1).

a) In normal individuals, before and after the administration of 500 mg per day of alpha-tocopherol for 10 days, we found the following values (table 1):

TABLE 1

1. Soggetto Nome	2. Tasso dei tocoferoli ematici	
	a. prima	b. dopo
L.F.	gamma 370 %	gamma 980 %
Z.A.	" 370 %	" 1210 %
C.T.	" 420 %	" 1740 %
O.A.	" –	" 890 %
M.T.	" 420 %	" –
S.U.	" 950 %	" 1760 %
G.V.	" 610 %	" 2350 %
F.P.	" 420 %	" –

Key:
1.= name of subject 2.= rate of blood tocopherols
a.=before b.=after

It is therefore certain that the dose and the manner of administration used by us lead, in man, to a precise increase of the rate of vitamin E in the blood. From this evidence it is also revealed that by subjecting to the same treatment individuals who have initially an identical blood concentration of vitamin E, clearly diverse values can be arrived at, which demonstrates a diversity in the character of the response to the addition of this vitamin on the part of individuals who still seem to metaboliz it in a like manner when they receive it in physiological doses. These diverse results can be explained by a different intestinal absorption or by a varied utilization of the vitamin. According to Beckmann et al. (2), the administration of 100 mg vitamin E to a patient is sufficient to heighten the blood tocopherol rate within 24 hours.

The values in a normal person reported by us agree with the evidence of the bibliography, values nonetheless notably broad (see Bicknell (3)), according to the researchers and the methods used, and the results of the tests by stress agree with the results communicated by Overman (4).

b) in the hyperthyroidal patient we found the following values, which we list, in table 2, alongside other data on their metabolism which can attest to the clinical diagnosis. (see next page)

c) in the hypothyroidal person we found the following values (table 3). (see next page)

TABLE 2

1. Ipertiroidei - Valori dei tocoferoli ematici				
a. Nome	b. Tocoferoli ematici (gamma %)	c. Metab. basale (variaz. %)	d. I.O.S.[1] (gamma % cc)	e. Colesterolo (mg % cc)
Novarino	230	+ 54	7,6	191
Moretti	140	+ 13	6,1	191
Lenti	350	+ 31	6,5	155
Deider	0	+ 36	6,5	245
Destro	250	+ 19	6,4	235
Gioberti	130	+ 24	6,4	230

Key:
1.= Hyperthyroids -- Values of blood tocopherols
a.= name b.= blood tocopherols c.= basal metabolism (variations
d.= serum organic iodine e.= cholesterol

TABLE 3

1. Ipotiroidei - Valori dei tocoferoli ematici				
a. Nome	b. Tocoferoli ematici (gamma %)	c. Metab. basale (variaz. %)	d. I.O.S.[1] (gamma % cc)	e. Colesterolo (mg % cc)
Pregliasco	500	- 3	-	-
Di Lorenzo	800	- 7	5,1	300
Robiola	650	- 10	5,1	250
Matich	580	+ 7	3,9	228
Viano	1630	- 6	3,2	-
Viano (dopo tratt. con tiroide)	450	+ 28	-	205
Pallanza	440	- 6	5,5	235
Biscaro	640	- 35	3,3	265
Alessandria	1050	-	3,8	275
Mortara	1425	-	5,2	260
Costero	95[illegible]	-	5,4	140

For key see table 2.

Thus it is evident that in the condition of hyperthyroidism one can as a rule observe a blood value of E lower than the normal averages, in hypothyroidism a value higher than the normal averages. Particularly instructive is the case of patient Viano, whom we were able to study in conditions of profound spontaneous hypothyrosis and then during the pharmacological cure; upon normalization of the thyroid function, the rate of E descended to one third of the value read in the preceding condition of hypothyrosis.

The data offered for the argument in the bibliography are scarce, but in sum consistent. As concerns hyperthyrosis it is admitted by most that the stimulation of the metabolic processes, characteristic of these patients, increases the velocity of utilization of vitamin E and therefore leads to a derletion of its deposits, plasmatic as well as tissue. Rapid temporary drops

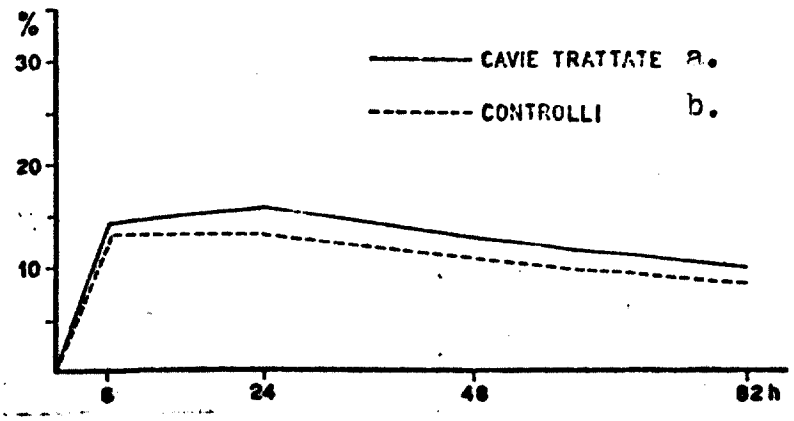
in the blood levels of E are observed in the case of infectious diseases and after acute fatigue (Urbach (5)).

Wheeler (6) observes that the addition of thyroproteins accelerates and increases mortality from avitaminosis E in the chick, while the addition of thiouracile retards it. Drill (7) attests that the addition of thyroid to food otherwise normal renders it insufficient, especially in its vitamin equilibrium. Tentori (8) has observed the degenerative muscular alterations of avitaminosis E appear, in those rats which were rendered hyperthyroidal through the administration of thyroid, several months sooner than they do in the controls. These bibliographical data are indirect: we have not found any direct bibliography concerning the blood level of E in the condition of clinical or experimental hyperthyrosis. As for hypothyrosis, Lemley (9) and other authors have observed that the plasma level of E is as a rule elevated in those physiopathological conditions which reveal elevated cholesterolemia. Thus Wechsler (10), Bensley (11) find a supernormal plasma level of E in the hypercholesterolemic diabetic; Darby (12) finds the same in hypercholesterolemia of persons of advanced age. On the other hand, in hepatopathies, an increased cholesterolemia is not always accompanied by an elevated level of E (Klatskir (13)); the latter would be elevated in cases of nephritis, not in cases of nephrosis (Popper (14)). Then there are contrasting voices: Chieffi finds an increase in blood tocopherol in the aged male, not in the female; Curcio (15) finds, in advanced age, an increase in the serum of cholesterol and carotenoids, but not of vitamin E.

II. HISTOLOGICAL AND FUNCTIONAL MODIFICATIONS IN THE THYROID INDUCED BY PROTRACTED TREATMENT WITH LARGE DOSES OF VITAMIN E

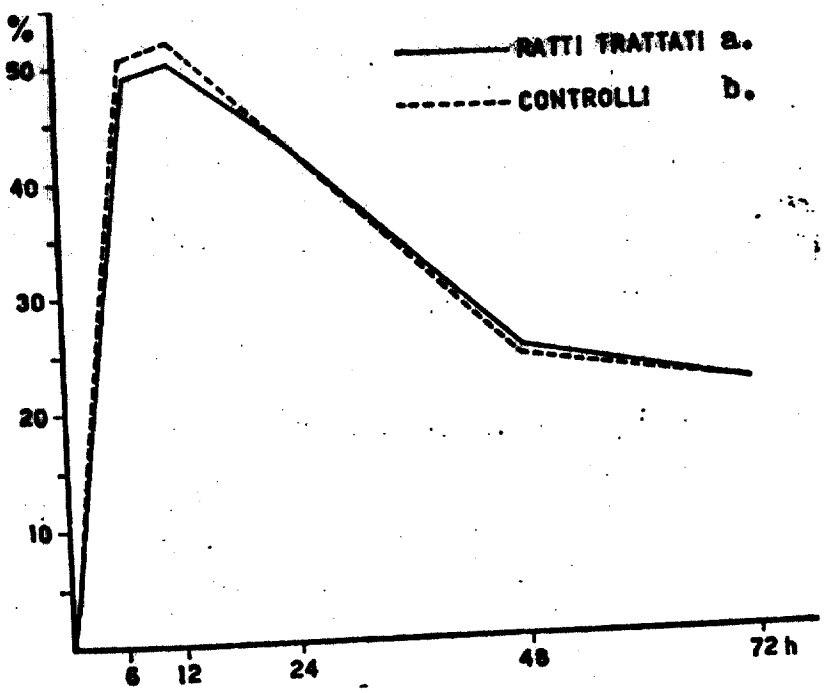
a) The histological research was conducted on the guinea pig, with the collaboration of Doctors Ferraris and Pansa.[1] A first lot of animals received 3 mg daily of alpha-tocopherol administered parenterally until a total of 50-60 mg was reached; other groups received vitamin E parenterally, and in greater quantities orally, in a dose of 13 mg per day (of these 10 were given orally), for 10 days. We did this because, according to some authors, the tocopherols administered parenterally follow metabolic paths different from those which are administered orally. So, in all the animals treated with vitamin E we observed

GRAPH NO. 1



an intense hyperemia of the thyroid, especially in those capillarie and precapillaries which are found in direct contact with the follicles. In the thyroids of the animals treated longer and more intensively, each follicle appears to be surrounded by a corona of capillaries full of blood: otherwise the histological report presents no morphological or color differences as compared with the controls.

GRAPH NO. 2



Micrometric measurings of the height of the cells were also carried out: the measuring of about 2000 epithelial elements of the thyroids of animals treated with vitamin E and of about 2000 epithelial elements of the thyroids of control guinea pigs revealed no appreciable differences. Both in the ones and in the others it is possible to recognize some follicles of lesser and others of greater amplitude, with practically equal frequency. The small follicles show cubical or cylindrical epithelia, the height of which varies between 8 and 16 microns (on the average 10-12 microns); the large follicles show cubical or cylindro-cubica epithelia, the height of which varies between 4 and 14 microns with an average value of 8-10 microns (figs. 1,.2, 3, 4).

In order to lend the histological analysis the most functiona significance possible, we turned to the autohistoradiographic method, testing the localization of radioiodine in the histological sections of the thyroid with the method of blackening of photographic emulsions. The localization of I^{131}, administered to the animal 24 hours before killing it, appears increased in the colloids as much in the treated animals as in the controls. The

The blackening, which with its intensity can give an idea of the accumulation of the radioelement, was shown, upon inspection, to be slightly more intense in the histological preparations of the animals treated with vitamin E: this nonetheless varies notably from one zone to another in the same thyroid (figs. 5 and 6).

TABLE 4

AVERAGE VALUES OF IODINE UPTAKE IN THE CONTROLS AND IN THE GUINEA PIG TREATED DURING VARIOUS PERIODS WITH VITAMIN E

Valori di captazione %		dopo 6 h	dopo 24 h	dopo 48 h	dopo 82 h	Osservazioni (dosi di Vit. E somministrate)
Cavie I Lotto	+ Vit. E	11,2	11,9	9,2	5,8	7 cavie (mg 15×15 gg) (per via parenterale)
	controlli	9,6	9,6	7,2	5,0	4 cavie controllo
II Lotto	+ Vit. E	17,8	17,4	13,8	10,0	14 cavie (mg 3 parenteralmente + mg 10 per os p. d. per 10 giorni
	controlli	16,4	15,0	12,8	8,5	9 cavie controllo
III Lotto	+ Vit. E	13,0	16,3	15,4	11,1	13 cavie (mg 14 per os) per 15 gg.
	controlli	12,2	13,3	10,7	9,1	7 cavie controllo
Valori medi	+ Vit. E	14,0	15,2	12,1	9,9	
" "	controlli	12,7	12,6	10,2	7,5	
Variazioni	percentuali	+ 10	+20,6	+18,6	+18,5	

A.= values of uptake B.= Observations (doses of vit. E administered)

Cavie = guinea pigs Lotto = lot Valori medi = average values
Variazioni = variations controllo = control dopo= after

Agreeing with these data are the values of iodine uptake. As it appears from table 4 and graph 1. the treatment with vitamin E in the doses and for the time given increases the thyroid uptake of iodine the guinea pig. We will verify the same phenomenon in man, but it is not present in the rat (table 5, graph 2).

TABLE 5

AVERAGE VALUES OF IODINE UPTAKE IN THE CONTROLS AND IN THE RAT TREATED WITH VITAMIN E

Valori di captazione %	dopo 8 h	dopo 12 h	dopo 24 h	dopo 48 h	dopo 72 h	Osservazioni (dosi di Vit. E somministrate)
+ Vit. E	49,2	50,3	42,9	24,7	21,2	10 ratti (mg 15 p.d. per os per 25 gg.)
controlli	50,7	51,8	42,8	23,8	21,3	10 ratti control.

For key see table 4.

b) In order to study the histological and functional behavior of the thyroid in the condition of avitaminosis E, we experimented with the rat. The animals received the test dose of radioiodine, and its uptake curve was then determined; then the animals were subdivided into two groups, one of controls, the other of experimental animals. The latter was kept in the same cage conditions as the latter, but was put on a diet with no vitamin E, no. 232 of Evans and Burr, which contains the salt blend of Osborne and Mendel.[1]

After a month and a half of this dietetic treatment we intended to examine the functional condition of the thyroid, even though supposedly the deposits of vitamin E in the rat are not exhausted until about three months of treatment with the above-name diet. We wanted to ascertain that the iodine uptake was practicall non-existent.

As security in our experiment, we also set up controls represented by animals kept on the above-named diet, but with vitamin E re-added in what was judged to be a physiological dose -- at least for a few months (Evans (16)) -- of 0.27 mg per head per day. These control animals also revealed a minimum uptake. Thus it was evident that the administered diet reduced the thyroid uptake of iodine by means of a factor that was not the lack of vitamin E. A calculation of the components of the diet led us to ascertain that the daily dose per head (15 grams) contained a quantity of about twenty gamma of iodine, a portion certainly exceedingly the physiological one per animal.

In table 6, the values of uptake obtained.

TABLE 6

CURVE OF THYROID IODINE UPTAKE IN THE RAT
10 MICROCURIES OF I^{131} PARENTERALLY

1.	Animale — 2. Tempi:	dopo 6 h	dopo 24 h	dopo 55 h	dopo 72 h
A.	Normale (20 ratti) dopo 50 gg di stabulazione a dieta normale	37,2	34,8	31,8	25,1 %
B.	Tenuto alla dieta di Evans e Burr N. 232, per 50 giorni (20 ratti)	9,9	6,4	5,3	5 %
C.	Come i precedenti, ma la dieta è stata riaddizion. di Vit. E (10 ratti)	6,9	5,3	5,1	4,7 %

Key:
1.= animal 2.= time
A.= normal (20 rats) after 50 days of penning on a normal diet.
B.= Kept on diet no. 232 of Evans and Burr for 50 days (20 rats).
C.= As the preceding, but with vitamin E re-added to the diet (10 rats).

[1] The diet was supplied for us by Dr. Piccioni of Brescia.

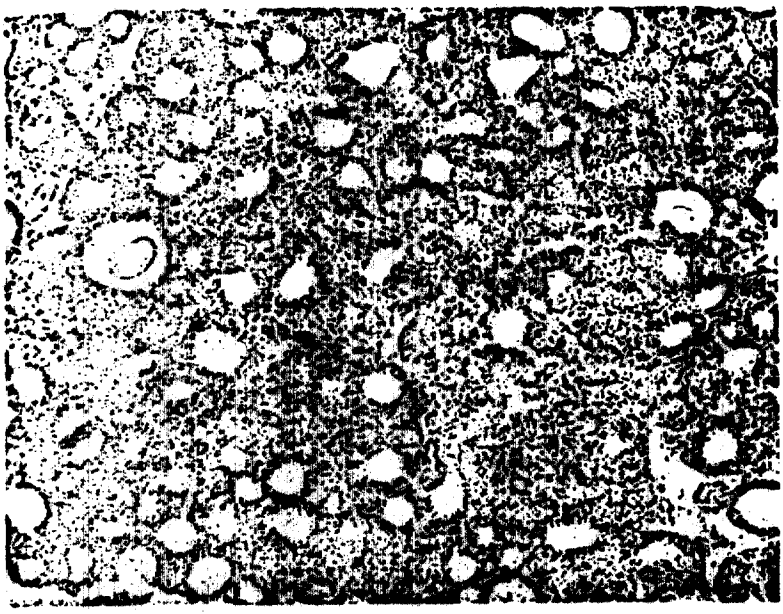


Fig. 1. Histological preparation of a normal guinea pig. Hematoxylin eosin coloration. Enlarged 30 times.

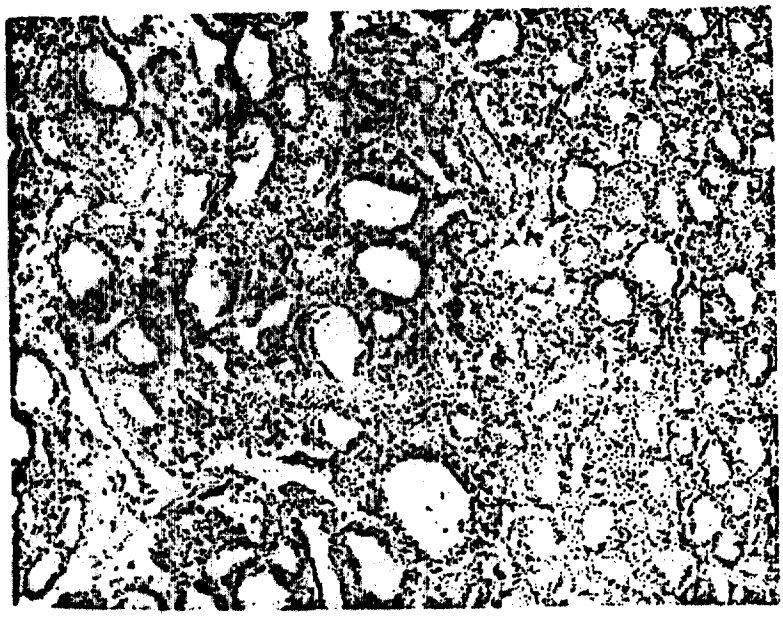


Fig. 2. Thyroid of a normal guinea pig. As above.

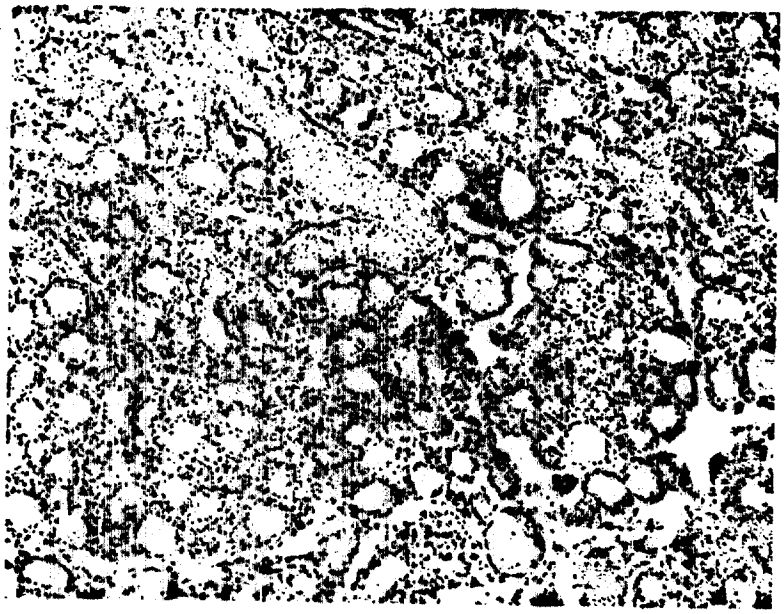


Fig. 3. Thyroid of a guinea pig treated with vitamin E. Notable hyperemia.

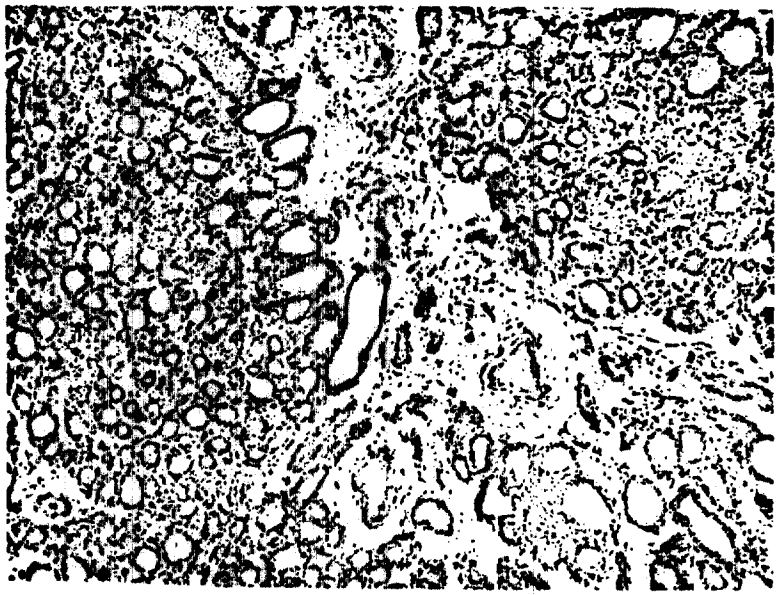


Fig. 4. Thyroid of a guinea pig treated with vitamin E. Hyperemia. Microfollicular picture.

Fig. 5. Autohistoradiography of the thyroid of a normal guinea pig.

Fig. 6. Autohistoradiography of the thyroid of a guinea pig treated with vitamin E.

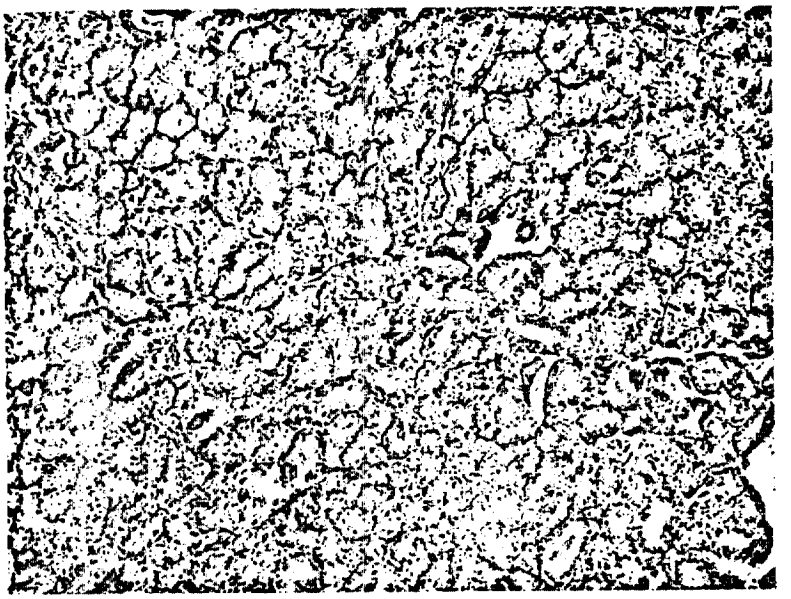


Fig. 7. Thyroid of a rat kept on a normal diet.

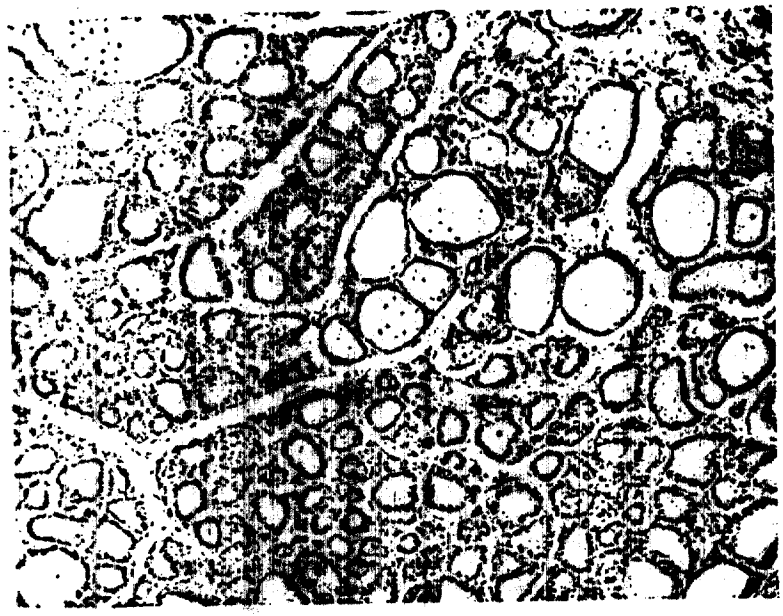


Fig. 8. Thyroid of a rat kept on a diet deprived of vitamin E, according to Evans and Burr.

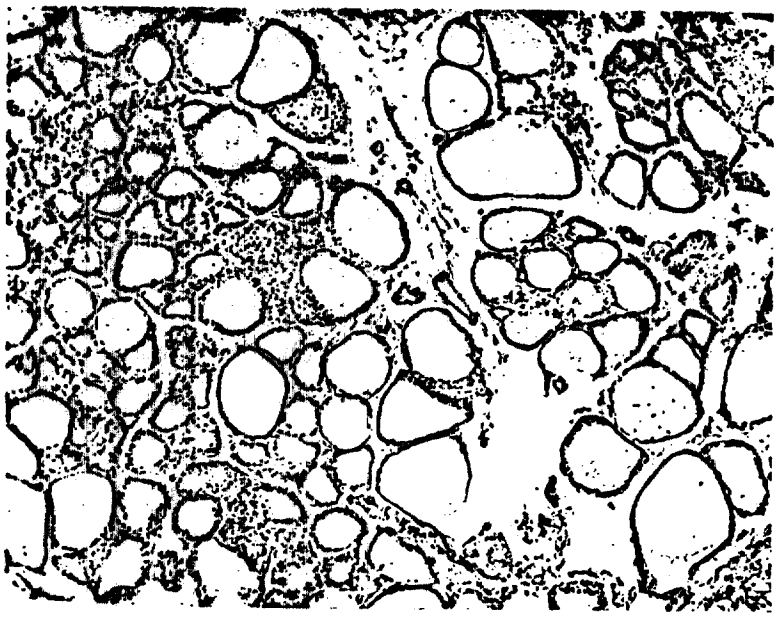
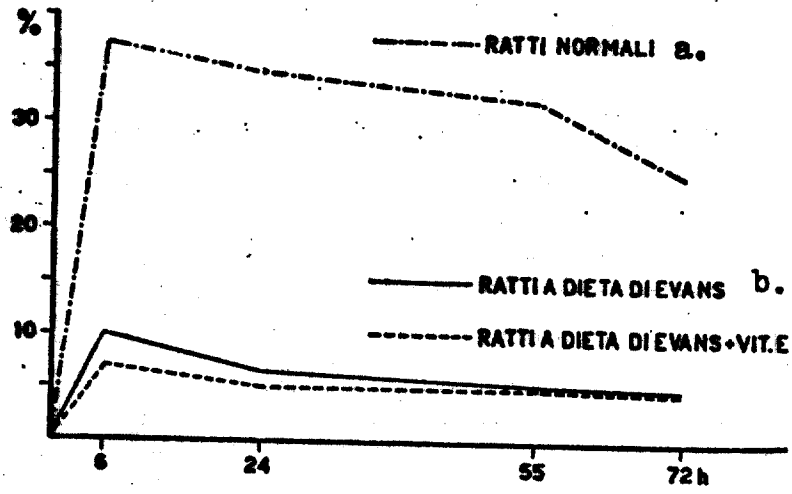


Fig. 9. Thyroid of a rat kept on a diet of Evans and Burr, but with vitamin E re-added.

The experiment is represented in graph 3.

GRAPH 3



a.= normal rats

b.= rats on Evar diet

c.= rats on Evar diet plus vi E

A certain number of animals was then killed and the histological picture of the thyroid studied. As appears in figures 7, 8, and 9, there is a clear attitude of repose of the thyroid of the animals kept on this diet (fig.8) even with a physiological dose of vitamin E re-added (fig. 9).

This is demonstrated by the presence of large follicles full of colloid, with a flattened epithelium, scarce vascularization, and development of the stroma greater than that in the animals on the normal diet (fig. 7).

Because of the limitation of the time given us, it was not possible for us to recommence research with a diet deprived of vitamin E and better balanced in salts: we can conclude that all the histological and functional experiments conducted with diets imperfectly dosed in their iodine content according to the needs of the animal preselected for the experiment, lead to results which lend themselves to erroneous interpretations. This is especially true for experiments on the structure and function of the thyroid, but also, because of the functional correlations between the endocrine glands, for every experiment conducted with such diets (figs. 7, 8, 9).

We have gathered the following bibliographical data on the argument. As early as 1938 Giesdoz (17) studied the modifications induced in the endocrine glands of the guinea pig by the administration parenterally of 6 cg vitamin E per day for 5 days. In the thyroid he observed histological characteristics denoting a hyperfunction. Sometimes these were only isolated zones of hypercolorability of the follicles; other times he observed an increase in the height of the follicular cells with copious endo-otricular desquamation, sparse colloid, abundance of intervesicular cellular

tissue. Paal (18) also speaks of histological pictures of thyroid stimulation upon intake of E.

On the other hand, Giberti (19) administered 4 mg alpha-tocopherol per day for a month to albino male rats; he observed neither structural nor weight modifications in the thyroid; he ascertained that this treatment does not impede the manifestation of the characteristic thyroid alterations of thiouracilics. Gitsch (20) finds no modification of the histological picture of the thyroid of the rat after administration of 3 mg vitamin E per day for a month: Bomskow (21) obtained analogous results.

Concerning the modifications induced in the structure of the thyroid by a diet lacking in E, we will record the experiments of Morgulis (22), of Escudero (23), Ruppel (24), Drummond (25), Teldorf (26), Zische, Mason, Byran (cited by Bicknell and Prescott (3)), all of whom find, in the case of a diet deficient in E, no appreciable alterations of the thyroid of the rat even when the histological examination reveals precise alterations of the muscles

Singer (27) and Barrié (28) find, in the rat put on a diet deficient in E for 12-18 months, hypoplasia of the thyroid with with an increase of the interfollicular connective tissue; involution of the thyroid was observable in the offspring of these animal as well.

Singer adopted for her research the diet of Evans and Burr, the high iodine content of which has already been mentioned.

Bomskow (21) also describes histological aspects of thyroid inactivation (follicles with a flat epithelium, full of colloid) in the baby rat kept on a diet deficient in E for not less than a couple of months; Scheider, Drummond, Sawada, authors cited by Butturini, have communicated similar data.

Lecoq (29) subjected rats to diet BR28 of Evans, deprived of vitamin E, for six months: at the end, the animal presents a certain esophthalmus and, in the thyroid, histological aspects of quiescence, constituted by the irregular volume of the follicles, the flat, cubic aspect of the epithelium, and the variable tingibility of the colloid.

According to Bomskow (30), the responsiveness of the thyroid to thyrotropic hormone would be less than the norm in animals deficient in E.

In a first group of experiments, Biddulph (31) observed that the weight of the thyroid of male rats kept on a diet deficient in E for more than six months assumed a weight double that of the controls: this does not occur in the females, in which the weight of the thyroid remains invariable.

In successive experiments, intended to find an explanation of the fact, he clarified the fact that the pathogenetic factor of this hypertrophy resides in a deficiency in iodine of the administered diet: the methods intended to deprive it of vitamin E also deprived it of iodine, so that the rats placed on a diet deficient in E received only about a quarter the amount of halogen as the controls. Pappenheimer (32) and Evans (33) also recall attention to the importance of the salt balance, and especially

the iodine content, of the diets lacking in vitamin E, so that the interpretation of the results is not a mistaken one. They recall, by the way, the judgment of Salter (34), that "the pituitary activity, as concerns thyrotropinic incretion, increases little by little as the iodine reserves decrease in the animal, and reciprocally it decreases with the increase of the latter."

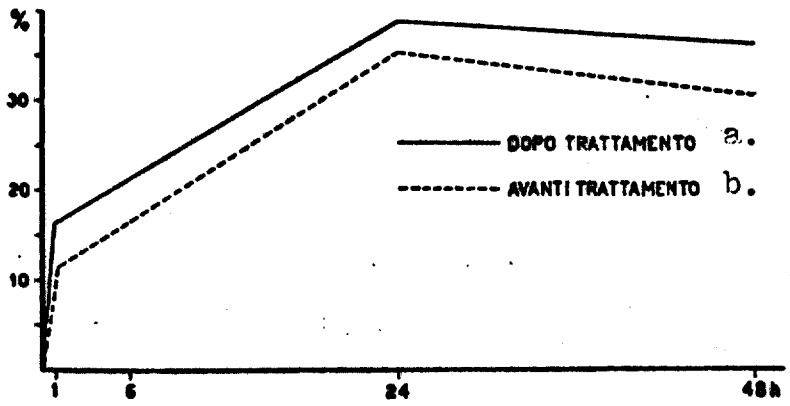
According to the experiments of Levine et al. (35), two gamma of iodine a day would be quite sufficient to maintain the normal structure of the thyroid in a rat weighing about 60 g. We have seen that the diet of Evans and Burr provides nearly ten times that amount! Therefore, we can attribute the pictures of thyroid involution observed in our rats kept on the aforementioned diet to an excessive intake of iodine with the diet: we are confirmed in this opinion by the fact that this involution is also observed when E is re-added to the food, and by the fact of the nearly non-existent iodine uptake of these quiescent glands.

III. MODIFICATIONS OF THE THYROID FUNCTION AND OF THE METABOLIC BALANCES MOST DIRECTLY GOVERNED BY THE THYROID, UPON ADMINISTRATION OF LARGER DOSES OF E

Twenty-six patients, of middle age, brought to the hospital for observation and recognized as clinically healthy, received 400-500 mg vitamin E per day for 9-10 days[1]: it was seen that this treatment succeeded in tripling the rate of blood tocopherols. The metabolic data determined before and after the treatment are shown in table 7 (see next page).

a) Thyroid uptake of iodine (see graph 4).

GRAPH 4



a.= after treatment
b.= before treatment

In 72% of the patients we observed a particularly distinct increase in the uptake values upon the first determination, when that is, the uptake phenomenon is detailed with the most evidence

[1] Synthetic alphatocopherol acetate, kindly placed at our disposal by Roche and Bracco, whom we sincerely thank.

because it does not yet appreciably influence the dismissing of the halogen from the gland. There are no bibliographical data on the question.

TABLE 7

PERCENTAGE DIFFERENCES BETWEEN THE ABSOLUTE VALUES OF VARIOUS METABOLIC DATA BEFORE AND AFTER TREATMENT WITH VITAMIN E (40-50 cg PER DAY, FOR 9-10 days)
NORMAL SUBJECTS

Name Nome	Age Età	M. B.	Capt. tir. I^{131}			I.O.S.	Colesterol	Glutatione		R.A.
			1 h	24 h	48 h			R.	O.	
1. M. Enrico	51	−19	+231	+ 22	− 8	+ 1	−20	+ 9	− 21	-
2. F. Maria	42	−10	+ 24	+124	+ 58	+10	+19	− 1	− 36	-
3. D. Adriana	20	+ 1	+ 19	− 27	+ 15	+13	+ 9	− 18	− 12	-
4. B. Anna	8	− 4	+303	+ 40	+ 23	+ 6	+ 6	+ 5	+ 26	-
5. B.Margher.	58	+16	+345	+112	+202	−17	+10	− 14	+154	-
6. R. Anna	61	−15	-	-	-	− 1	+30	− 17	+383	-
7. T. Anna	20	− 5	+ 35	− 16	+ 52	+27	+34	+ 3	+216	-
8. B. Carlo	57	−19	-	-	-	+ 5	−15	− 11	− 47	-
9. P. Leone	55	− 4	-	-	-	-	+10	− 9	+ 88	-
10. P. Elena	16	± 0	+ 42	+ 5	+159	+ 2	+43	−11	+143	-
11. B. Sergio	53	± 2	+ 85	+ 1	+ 29	+ 5	− 9	− 15	+ 36	-
12. B. Franco	48	± 0	− 6	+ 8	− 10	-	-	− 5	+ 79	-
13. G. Pietro	55	− 5	-	-	-	− 4	+33	− 9	+292	-
14. F. Maria	61	− 2	+ 10	− 18	+ 25	− 1	+40	− 41	− 14	-
15. V. Paola	28	− 4	+220	+ 48	+ 24	+10	-	+ 38	− 44	-
16. M. Elide	24	− 9	− 25	− 74	− 66	+ 4	−14	− 4	+ 4	-
17. F. Etelino	38	+ 3	-	-	-	+14	−24	− 62	− 5	-
18. O. Attilio	29	± 0	− 25	± 0	− 19	-	− 9	− 19	+ 13	+16
19. R. Giovan.	25	+ 6	+ 12	− 50	− 39	+18	− 5	− 31	− 19	+ 4
20. M. Frances.	47	− 3	− 11	− 18	− 14	− 9	-	± 55	+854	−37
21. L. Ferdin.	30	+ 1	− 76	+ 26	+ 20	± 0	+ 3	+172	+110	− 2
22. B. Giovanni	31	−10	+ 42	+ 62	-	+10	+46	− 1	+ 72	+45
23. R. Luciano	27	+ 7	− 32	− 17	− 6	± 0	− 6	+ 6	+150	− 3
24. C. Teresa	17	− 7	+276	+ 40	+ 12	+ 6	−26	− 1	+ 25	+63
25. Z. Albina	29	± 0	+422	+ 23	+ 12	+ 3	+ 5	− 19	+167	− 2
26. M. Teresio	30	− 4	+100	+ 17	− 12	+16	+73	− 15	+ 1	− 4
Medie delle variaz.		−3,2	+ 95	+ 15	+ 23	+5,1	+10	−4,8	+100	+8,8
Percentuale dei casi										
Varianti in più		27	72	62	60	69	60	23	70	44
Varianti in meno		58	28	33	40	22	40	77	30	56
Invariati		15	-	5	-	9	-	-	-	-

1.= averages of the variations
2.= percentages of the cases
3.= most varying
4.= least varying
5.= invariable

b) The level of serous organic iodine. This value also reveals a slight increase in 70% of the cases. Nonetheless, since the average deviation is only one of +5%, we must keep in mind that in the determining of the organic iodemia rate, there may compete factors which can modify it in an opposite sense:

e.g. the thyroid incretion of the hormone and the peripheral consumption of it. Jacono (36) did not observe any appreciable modifications in the serous organic iodine after administration of 300 mg vitamin E per day for three consecutive days to normal or hypometabolic individuals, while he found a slight decrease in it when the same treatment was applied to hyperthyroid individuals or hypermetabolic ones. He attributes this modification to an inhibited incretion of the thyroid.

c) Basal consumption of oxygen. This decreases in 58% of the cases; nevertheless the average variation is very slight (-3.2% of the initial values). The meaning of the deviation finds confirmation in the blibliographical data. The administratic of vitamin E to a normal rat decreases its consumption of oxygen, most markedly at the expense of the combustion of the muscles, (Zierler (37), Markees (38)), while the lack of this vitamin leads to an increase in the respiratory metabolism, whether of the muscular tissue (Vernar (39)) or of the entire animal (Verzar (40), Wood (41), Goettsch (42)), but not of the hepatic tissue (Kaunitz (43)). In experiments which go back to 1939 Bomskow (44) found that the addition of vitamin E to guinea pigs normally nourished does not provoke any modifications in the consumption of oxygen; Prange (45) and La Grutta (46) found analogous results. Markees (47) on the other hand, finds a definite if temporary decrease in the basal metabolism of the experimental animals and attributes it to a slowed down peripheral exchange owing to the action of the vitamin.

Zierler (48) observes that the administration of alpha-tocopherol to rats determines a depression in the basal consumption of oxygen, a depression of the respiration of the skeletal muscle but not of the liver, nor of the brain; Kaunitz (49) affirms that the basal consumption of oxygen increases notably in rats deprived of E before the signs of muscular dystrophy even appear.

Hottinger (50), adding 90 mg vitamin E to the diet for 2 or 3 days, observed, in a third of the subjects, a decrease in the basal metabolism and a decrease in the specific dynamic action both of the food and of the glycocoll; Heisen's (51) results are concordant with this. Baroni and Casa (52) administere more than ten centigrams of E per day for a period of about a month to men, obtaining thus a decrease in the basal metabolism, consistently more evident as the treatment was more protracted. In normal subjects and in hyperthyroidal patients Jacinto and Curro (53) obtain a decrease in the basal metabolism of between 10 and 20% as soon as three hours after the administration of 300 mg vitamin E. Jacono and Alessandro (36), administering 300 mg vitamin E for three days, observe a decrease in the basal metabolis though only in hyperthyroid or hypermetabolic patients. Schmid (54) finds in this vitamin a slight and temporary influence on the basal metabolism, though, in sum, in the sense of a depression, most evident in hyperthyroid patients.

Contrasting are the data of Roncalli and Meneghini (55), who find in different patients an increase in the basal consumption of oxygen after the oral intake of vitamin E.

Thus it seems certain that the intake of vitamin E, in addition to the normal food, causes a diminution in the consumption of oxygen, even though slight and not constant, in both the healthy and the hypermetabolic individual. The modifications induced by E in this metabolic moment would seem therefore to suggest a hypofunctional condition of the thyroid, incontrast thus to the data deduced from the histological examination of the viscera and from the data of iodine exchange. This leads us to attribute the diminution of the gas exchange after the introduction of vitamin E to a modification induced in the peripheral tissue metabolism, rather than to a hypofunction of the gland. Antognetti (56) suggests that a possible involvemen of the glycidic utilization in the periphery and disturbances in the muscular creatinic metabolism may be causes.

d) Cholesterolemia. This value was determined by the method of Urbach: the values of cholesterolemia increase in 60% of the cases, though in a very slight way, so that the average deviation does nor surpass +10%. It mig..t be asked whether the introduction of 5 grams of alphatocopherol in ten days may not interfere in the reaction of Libermann, which is not strictly specific for cholesterin (Lipschutz): without entering into the chemical aspect of the problem we will observe that such a possibility seems to be excluded by the fact that in the remaining 40% of the patients we observed a decrease of blood cholesterol, and that, in case of increase, this appears to be variable in intensity from one patient to another. In experiments on schizophrenics, Rossen (57) observes that the introduction of alphatocopherol heightens the level of cholesterol and of the fatty acids in the blood. Bottiglioni et al. (61) also find an increase in cholesterolemia after administration to the patients of 600 mg vitamin E per day for a period of 7-10 days. Bensley (58), administering vitamin E to diabetics, does not find that it raises the cholesterolemic rate. Marx (59) does not find an increase in cholesterolemia in the rat after introduction of E; on the other hand, Manuel (60) does establish it in the rabbit. Schwarz does not find modifications of the cholesterolemia in man after 12 hours following a single injection of 600 mg alphatocopherol, and Bonati does not find any upon the parenteral administration of 300 mg alphatocopherol per day for six days.

We have mentioned above how generally one observes an increase (spontaneous) of tocopherolemia in those conditions in whic there is a heightened cholesterolemia; we have confirmed this in cases of hypothyroidism.

Thus in cases of hypervitaminosis E, cholesterolemia changes its place (thus like the basal consumption of oxygen) in that same way in which it changes in hypofunction of the thyroid.

e) The alkali reserve. This we determined with the method of Van Slyke and Cullen; we observed, in man as well as in the animal (table 8) variations of the initial values in both directions upon treatment with vitamin E: viewed in sum, there is a slight tendency toward increase.

This evidence finds support in the bibliography: vitamin E woubd seem to have an alkalizing effect; the intravenous injection of it increases the alkali reserve of the plasma (Lecoq (29)).

TABLE 8

ALKALI RESERVE IN THE PLASMA OF THE GUINEA PIG

Guinea pigs on normal diet (n. 9)

alkali reserve: Average value: cc 48.1 (CO_2/100 cc plasma)
Extreme values: cc 40 - cc 62.6

Guinea pigs on diet with vitamin E added (15mg orally for 15-30 days) (n. 10)

Alkali reserve: Average value: cc 51.2 (CO_2/100 cc plasma)
Extreme values: cc 40.3 - cc 66.4

Average increase of alkali reserve in the guinea pigs on diet with added vitamin E: +6.6%

If we consider that which occurs in the course of the functional thyroid deviations, we find that as a rule there is an increase of the alkali reserve in the conditions of hypothyrosis, a decrease in hyperthyrosis.

f) Glutathionemia. We determined the blood glutathione with the method of Woodward and Fry: Dr. Della Beffa collaborated with us on these experiments. In man, in the majority of cases (70%), we observed a slight decrease of the reduced glutathione rate (average deviation -5%) and a just as frequent increase of the oxidized glutathione (average deviation +100%). In the guinea pig the behavior was somewhat different (table 9) in that we observed an increase in both the fractions -- a slight one in the reduced glutathione (+26%), a distinct one, of more than 100%, in the oxidized glutathione.

TABLE 9

BLOOD GLUTATHIONE IN THE GUINEA PIG

7 guinea pigs on normal diet:

Reduced glutathione: average value: 24.08 mg per 100 cc blood
extreme values: from 16.95% mg to mg 38.46%

Oxidized glutathione: average value: mg 5.11 per 100 cc blood
extreme values: mg 2.60% to mg 8.80%

10 guinea pigs on diet with added vitamin E (15 mg orally for 15-30 days):

Reduced glutathione: average value: 30.47 mg per 100 cc blood
extreme values: mg 17.93% to mg 52.16%

Oxidized glutathione: average value: mg 10.45 per 100 cc blood
extreme values: mg 3.26% to mg 18.90%

The reduced glutathione increases by 26.5%; the oxidized increase by 104.5%.

We found disagreement in the experiments of Bottiglioni (61), who observes that the introduction of vitamin E into the human system, whether rapid or protracted, leads to a circular increase of both the parts of the glutathione, the variations of the oxidized share being nonetheless inconstant, both as regards direction and as regards intensity.

It has been noted (Barbaro-Forleo (62)) that the increase in the oxidized fraction (-S-S) of this fermentum can be observed in conditions of increase in the vagal tone, in alkalosis, in those hormonal and metabolic conditions which in general favor anabolism, as for example in the introduction of insulin, and, as for disfunctional thyroid conditions, in hypothyroidism (Giordano and Battistini; Barbaro-Forleo; Cattaneo; Rivoire and Bermond). In contrast to this are the conditions of intensified catabolism, e.g. in the introduction of adrenalin or thyroxine (Inglessi (63)), in clinical hyperthyrosis and in experimental hyperthyrosis, in which there is observed a definite drop in the oxidized fraction. In the hyperthyroid patient Della Beffa (64 found a drop in the oxidized glutathione rate to 80% of the normal, without notable variations in the reduced fraction. Volterrani (65 treating normal individuals with thyroxine or with thyrotropic hormone observed, in nearly every case, a drop in the oxidized fraction of glutathione.

This ascertainment leads us to consider one of the characteristic moments of the action of the thyroid incretion on the one hand, and of the tocopherols on the other, which is to say, their interference in the process of oxidation in the tissues. The thyroid hormone (and this is what we refer to when we speak of "thyroxine") seems to unite in the periphery with the colloidal constituents of the cytoplasm in order to form an enzymatic system (thyrenzyme) which would have the effect of strengthening the normal catalysts of cellular respiration (Dye; Rabinowitch; Cohen). Wingler maintains that the function of the thyroid hormone is that of accelerating the oxidating processes of the cell, though not in all the tissues (not in the thyroid tissue). Salter(66) maintains also that thyroxine intervenes in some intracellular oxidating processes, increasing the consumption of oxygen; but not in the thyroid tissue.(Galli Mainini (67)). And thus in hyperthyrosis there is observed a definite increase of the activity of the succinyl-dehydrolase, cytochrome, cytochromooxidase systems (Galeone (68)) and the same is observed in the case of deficiency in vitamin E. (Tipton (69)).

In contrast, the tocopherols, and of these most predominantly, according to Kunkel (70), alphatocopherol (according to others -- Stern (71) -- less distinctively) acts as a "non-specific general antioxidant in all the tissues" (Hickman (72)).

It is not included in our task to provide instruction on the analysis of the antioxidant activity of vitamin E, how it is manifested, and the problem of whether it represents a specific biological action or merely an aspecific action correlated with its physical structure; on this aspect we call attention to the works of Mattill (73), Dam (74), and Harris(75).

We are limiting ourselves here to reporting that the anti-

oxidant activity of vitamin E is manifested, at least in part, in the inhibition of oxidative fermenta (peroxidase, catalase, ethyl alcohol dehydrogenase) (Tappel (76), Weinstock (77)).

Artificially obtained complexes of alphatocopherol with protein, such as to render the vitamin soluble in the presence of enzymes without having to take recourse to its phosphoric ester (which, perhaps, is not a normal part of the metabolism of the vitamin), demonstrate their capacity to inhibit by 25% the activity of the succino-oxidase system (Ames (78)). Hummel (7 and Boyer (80) have also verified this inhibiting action on the succinyl dehydrogenase, though they did conduct their experiments with the phosphoric esters of the tocopherol. Jacobi (81) has recently defended the concept that the inhibiting action of E on the succino oxidase system is biological and specific, and maintains that the intensified succino-dehydrolase activity of this system in the case of lack of vitamin E (Houchin (82)) is directly attributable to this very lack. B. Connor et al. find that substances with antioxidant action (diphenyl-para-phenylendiamine) might act in stead of vitamin E in that activity which has earned it the name of the "fertility vitamin".

Alphatocopherol could also inhibit lipoxidase, an enzyme wit an anti-oxidant influence on vitamin A and its carotenes (Holman (if one accepts the presence of an "antithyroidal" action of vitamin A (see bibliography, Morgano(84) and Travia(85)), one can see in this protective action another moment of antagonistic action which vitamin E seems to reveal toward the thyroid function. Besides this, alphatocopherol might inhibit the oxidation of the insaturate fatty acids, interceptingthe oxidative reaction on the level of the formation of the peroxides (Tappel (86, 87)).

Finally, according to some (Michaelis (88)), the same vitamin E might participate in a system of oxidoreduction, insofar as the tocopherols, easily oxidizable substances, can be oxidized (dehydrogenized) in a quinone, which contains the opening of their cyclical structure in a position of 1-2. But since this structural modification would be irreversible and would deprive the tocopherol of every vitamin activity, we maintain that this does not occur in the normal metabolism of E, as Riedner also affirms on the basis of experiments mixing marked alphatocopherol with radiocarbon.

Admitting therefore that vitamin E might possess in some intrinsic capacity and some form of biological action an antioxidant acitivity in the tissues, it is possible to recognize, in the increase of the oxidized fraction of glutathione following large doses of vitamin E, the consequence of slowed down processes of dehydrogenation (oxidation). This interpretation seems justified by the reported opposite situation of a decreased rate of oxidized glutathione when the peripheral combustions are stimulated (hyperthyrosis). In agreement with this concept of slowed down peripheral combustion following the introduction of E in large doses are the data on biochemical values which we studied: the decrease in the basal consumption of oxygen, the increase in blood cholesterol, the increase in the alkali reserve. Whatever may be the factor of these biochemical deviations observed upon the addition of vitamin E to a normal diet, it is evident

that this occurs in the same way as in hypothyrosis. In other words, we can say that the introduction of vitamin E in excess leads to a biochemical-metabolic situation comparable to that of a "peripheral hypothyrosis" (Somatic hypothyroidism, Starr (89))

On the other hand, the direct data concerning thyroid function attest to an increase in the function of the gland upon the introduction of vitamin E: hyperemia of the viscera, increased iodine uptake, increase in the circulating serous organic iodine.

We were therefore able to outline the hypothesis that the introduction of vitamin E places the organism in a condition equal to that of a "peripheral hypothyrosis" (see above), and, as a consequence of that, reveals a "reactive thyroid hyperfunction The introduction of this vitamin in excess therefore alters the functional and metabolic moments examined in the opposite sense in which they are modified by the introduction of thyroxine: this hormone, in fact, can determine a "peripheral" hyperthyroidism with definite notes of anatomo-functional repose of the thyroid.

Galeone (l.c.) called attention to the affinity of some biochemico-enzymatic, metabolic, and clinical aspects in the lack of tocopherol on the one hand and in thyroxine intoxication on the other. In the one condition as in the other, in fact, there can be observed marked increases in the consumption of oxygen and, parallel to this, in the succinyl oxidase activity of the striated musculature, as well as modifications of the creatine metabolism and neuromuscular and cardiac modifications which are partly immeasurable.

Thus deficiency in E leads, at least in some animal species, to a muscular dystrophy, to a decrease in the muscular creatine, and to intense creatinuria: miopathy and creatinuria, though they may be present in either hypo- or hyperthyroidism, are more frequent in the second of these diseased states (Mackenzie (90)).

Hottinger finds that by administering 12-18 mg of alpha-tocopherol daily to children, their physiological creatinuria decreases; thyrotoxic creatinuria also decreases upon the introduction of vitamin E (Butturini (91); Blummer; Chretien (92)). In the experiments of Braunsteiner (93), the increase in the consumption of oxygen which appears in the isolated muscle of the rat upon treatment with thyroxine is reduced by a preceding administration of vitamin E.

Both in the case of vitamin E deficiency and in the case of hyperthyrosis, there are alterations in the muscle of the processes of phosphorization, with the involvement of the adenosintriphosphate cycle, phosphocreatine (Hummel (94)).

Mazzullo (95) and Mosetti (96) find water retention upon the introduction of E, while as is known the thyroid preparations increase the elimination of water (Enninger; Volhard).

The introduction of E details a complex action in the glycidic metabolism, one that is contradictory in some of its aspects, but which is, in its general outlines, insulin-like (Butturini (91)), therefore opposed in many points to the effect of thyroxine, which we could say is, in its general nature, diabetogenic. In the case of lack of vitamin E, the glycogen in the muscles, the heart and the liver decreases (Progneri (97));

treatment with toconherol is capable of reducing hyperglycemia of thyroxine in the rat (Caltabiano (98)), as well hepatic glycogenolisis of thyroxine (Teodoro (99)).

Fischer (100) affirms that toconherol sodium sulphate impedes the typical action of thyroxine of stimulating the metamorphosis of the tadpole.

According to Underhill(100), in the case of vitamin E deficiency, there is a decreased formation of thyrotropic hormone with intensified alterations of the eosinophilic cells of the hypophysis: however, the relevant literature is extremely vast and contradictory (Nelson (102); P'an (103)).

It was therefore consistently shown by the data of the biological experiment that the treatment of hyperthyroidism with large doses of vitamin E can be attempted, and that a possible pharmacological activity of this vitamin in that field was tested, especially when the physician had neither synthetic antithyroids nor radioiodine at his disposal. Here are the results of these attempts.

Cima (104) finds that the introduction of vitamin E does not modify in any way the esophthalmus induced with the thyrostimulant hormone or with the thyrodectomy of the guinea pig.

Williams (105) finds no appreciable therapeutic effect of vitamin E on human hyperthyrosis, while Garcia (106) obtained good results in the treatment of three patients. Heisen (l.c.) obtained a decrease in the basal metabolism and an improvement of the general condition in six hyperthyroid patients. Giberti (19 administered a gram of vitamin E orally or parenterally per day to 11 ; he obtained a distinct decrease of the basal metabolism and sometimes a decrease of the thyroid volume. Because of the large doses used, he believes this could be due to a pharmacological and not a vitamin action of E: he judges that the results can be attributed to a peripheral anti-oxidant and antithyroxine action of alphatocopherol.

SUMMARY

I. The blood level of alphatocopherol in a human adult on a normal diet is, according to our results, 770-950 gamma per 100 cc. In the hyperthyroid patient it reveals below normal values, in the hypothyroid patient above normal values.

II. The daily introduction of 500 mg alphatocopherol for about ten days induces, in the normal individual, metabolic conditions like those which are found in cases of hyperthyrosis (decrease of the basal metabolism; increase of cholesterolemia; increase in the oxidated part of glutathione: increase in the alkali reserve) and simultaneously, signs of functional activation of the thyroid (increase in iodine uptake; increase of serous organic iodine).

In the guinea pig, large doses of vitamin E provoke thyroid hyperemia and increase in the fixation of iodine.

III. We propose the hypothesis that alphatocopherol performs an action in the periphery which is opposed to that of thyroxine, or anti-oxidant, determining a reactive hyperfunction of the

thyroid.

IV. The anatomo-functional experiments conducted with a diet deprived of vitamin E are not reliable if the iron content of the diet is not balanced according to the necessities of the animals in the experiment.

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ENDOCRINOLOGY: The Comparative Effects of α-tocopherol , Estradiol and Progesterone on the Female Genital Tract of the Golden Hamster.

by Jean-Claude Czyba, presented by Robert Courrier

In the golden hamster whose ovary has been removed a dose of 10 mg of vitamin E for 7 days exerts an estro-progesterone effect manifested by the obtention of microscopic traumatic deciduomas and the production of mucus in the vaginal epithelium. At the daily dose of 100 mg the estrogenic effect becomes predominant and causes stratification and keratinization of the vaginal epithelium.

The intervention of vitamin E in pregnancy conditions has been currently accepted since the first observations by Evans and Bishop who noted a decrease in the implantations in the female rat deficient in vitamin E and in the observations of Varangot who found an increase in the proportion of tocopherol in the blood during pregnancy. Different theories have been proposed to explain the beneficial role of vitamin E in the maintenance of pregnancy equilibrium.

In the opinion of Staehler and Hopp, vitamin E used alone exerts a progesterone-imitating action resulting in the formation of a serrated uterus in the female rabbit which has not reached puberty . This result was not found, however, by Kehl and Douard who believe that vitamin E only has the role of increasing the potency of the progesterone. Furthermore, tocopherol administered in strong doses did not enable to induce the formation of an experimental deciduoma in the castrated female rat.

According to Albrieux et al., on the other hand, α-tocopherol supposedly has an estrogenic property, since when administered to the female rat which has not reached the age of puberty it causes the vagina to open and when given to the adult castrated female rat it causes estruation. This estrogenic effect has not been found by other authors

Lastly some authors believed that vitamin E had androgenic properties, that it stimulates hypophyseal gonadothropic secretions, exerts a gonadothropic effect in the animal whose hypophysis has been removed , directly stimulates the ovarian follicle and sensitizes the endometrium to the action of the sex hormones.

The examination of these contradictory data prompted us to study the hormonal effect of vitamin E in the female golden hamster. In this species, in effect, the vaginal epithelium is particularly sensitive to the action of the sex hormones and the deciduation of

the endometrium can be produced by small doses of progesterone and is only impeded by large doses of estrogen.

Fig. 1 . The vaginal mucosa of a castrated hamster. Two-layered cube-shaped epithelium.

Fig. 2 The vaginal mucosa of a castrated hamster given a daily dose of 1 milligram of progesterone. Two-layered cube-shaped epithelium.

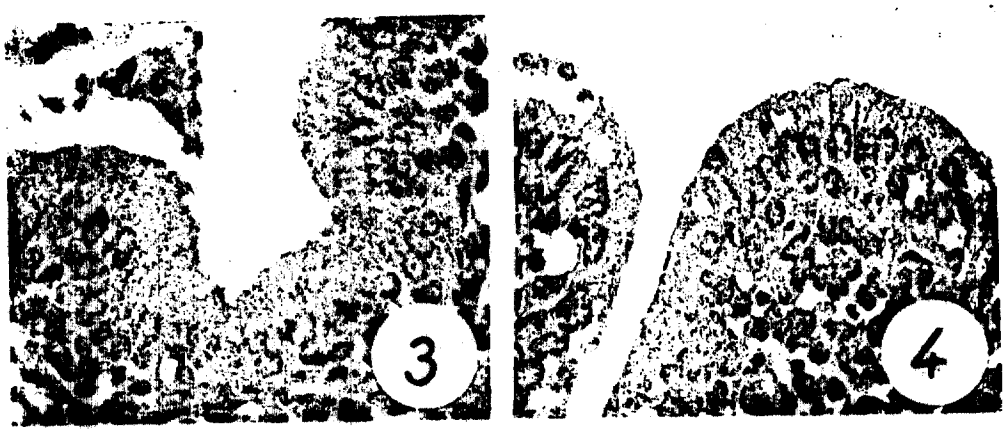

Fig. 3. Vaginal mucosa of a hamster on the 12th day of gestation. Mucified cylindrical epithelium.

Fgg. 4. Vaginal mucosa of a castrated hamster given vitamin E at the daily dose of 10 mg .
Mucified cylindrical epithelium

Materials and methodology. We used 25 adult females aged from 6 months to one year. All of the animals, with the exception of one female in gestation) underwent a bilateral ovariectomy one week prior to the start of the experiment.

In the first group twelve females were given daily injections of d-ℓ-α - tocopheron in oily solution. At the time of the first injection a thread of cotton was of a cornu of the uterus. The animals were sacrificed on the seventh day of treatment and we performed histological examination of seriated sections of the traumatized uterus and cuts made at the third posterior level of the vagina.

After analyzing the initial results we resumed our experiment on a group of six hamsters increasing the daily doses of vitamin E to 100 mg.

In a third group consisting of seven animals two females did not receive any treatment after castration, two animals received daily injections of 10 µg of benzoate of estradiol, two others were administered progesterone at the daily dose of 1 mg, and, lastly, one non-castrated female was sacrificed on the twelfth day of gestation. This procedure was inspired by the research conducted by M. Klein on the hormonal conditioning of the structure of the vaginal epithelium in the golden hamster.

Results. At the vagina of the animals of the third group we found the data gathered by M. Klein: epithelium composed of cube-shaped cells resting on a layer of flattened basal cells in the male castrated hamster (fig. 1) and in the castrated female treated with progesterone alone (Fig. 2); completely mucified mucified cylindrical epithelium on the twelfth day of gestation (figure 3); stratified and keratinized epithelium in the castrated female administered estradiol (fig. 5).

In the animals given a daily dose of 10 mg of vitamin E we observed a significant hypertrophy of the endometrium. The chorion is the site of a fairly large edema. It is excessively vascular , rich in cells and contains numerous glands. The uterine epithelium is composed in several places of various cell layers and mitoses occur there frequently. A small decidual reaction occurred in five females. The vaginal epithelium was formed of a layer of mucified cylindrical cells (Fig. 4).

In the animals given a daily dose of 10 mg of vitamin E the uterus was also hypertrophic but the chorion contained more vessels and fewer glands than in the females of the preceding group. The uterine epithelium presents a degenerative appearance; numerous cells are in the process of being eliminated. We did not observe any decidual reaction. The vaginal epithelium was multi-layered, lined, keratinized, lined on the outside with residue from mucified cells (fig. 6).

Comments. Vitamin E at the daily dose of 10 mg permitted us to carry outsmall experimental deciduomas. Thus it has a progesterone-

imitating action of a slight extent , but the appearance of the enometrium and above all of the vaginal epithelium alsq call to mind estrogenic activity. The mucification of the vaginal cells in the golden hamster can be observed during gestation or, in the castrated female, after the combined administration of progesterone and estrogen.

Vitamin E, when given at the daily dose of 100 mg has not been able to achieve the conditioning of a deciduoma and the structure of the vaginal epithelium reveals the predominance of the estrogen effect.

We think that we can reach the condlusion that in the castrated female golden hamster α-tocopherol exerts an estro-progesterone effect when administered at a dose of 10 mg daily. When the daily dose is increased to 100 mg the estrogen effect becomes predominant.

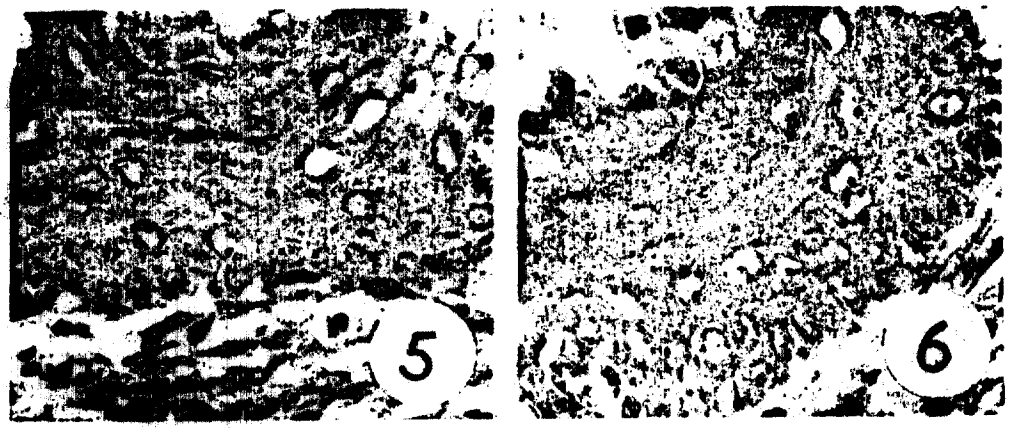

Fig. 5. Vaginal mucosa of the castrated male hamster treated with benzoate of estradiol at daily dose of 10 µg.
Epithelium lined, stratified, keratinized

Fig. 6 Vaginal mucosa of the castrated hamster male treated with vitamin E at daily dose of 100 mg. Epithelium lined, stratified, keratinized covered with residue of mucified cells.

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Physiology

THE EFFECTS OF EXCESSIVE AMOUNTS OF VITAMIN E (HYPERVITAMINOSIS E) ON THE GOLDEN HAMSTER

by J. C. Czyba *

Numerous studies have stressed the importance of the role played by vitamin E in the phenomena of reproduction. The work done in the male of different species (the rat and mouse essentially) have shown that the lack of vitamin E causes testicular atrophy with degeneration of the semen-producing tubes . It is, however, not possible to indicate whether the avitaminosis exerts an effect on the gonads or whether it brings about a disturbance in the hypophyseal secretions.

In contrast to this, however, the research which has been conducted on the action of heavy doses of vitamin E on the testicle and in particular on spermatogenesis has been relatively rare[1]. The results of the studies which have been conducted on this question are negative or not very conclusive and, furthermore, the authors observed a slight stimulation in spermatogenesis.

After having shown that vitamin E exerts in the golden hamster female, castrated, an estrogenic or estroprogesteronic effect according to the doses administered[2], we undertook the study of the effect of hypervitaminosis in the male of that same species. Our first results concern the genital system.

Materiel and methods. We used fifteen male adult hamsters about 6 months old and of the same weight. The animals were placed into several groups and each day they were given, by subcutaneous injection, 0.75 cubic centimeters of oil containing or not containing vitamin E at the dosage of 75 mg**. Eleven hamsters were treated with vitamin E and four other hamsters, used as the reference group, were only administered the excipient. The animals were sacrificed after receiving a treatment lasting from 8 days to one month. The two testicles were weighed and we took a testicle, the epididymis and a seminal vesicle for histological examination.

Results. The results are shown in part in the table below. At the time of the sampling the testicles of the animals which were treated seemed to be atrophied and in all of the cases the weight of the testicles of the animals treated was clearly below the weight of the testicles of the untreated control group of animals. The histological examination showed that the spermatogenesis occurred normally in the 4 control animals (fig. 1) but that it was more or less disrupted in the other males. In most of the seminiparous (semen-producing) tubes the evolution of the line terminates at the stage of auxocyte or spermatid and the lumen is deprived of spermatozoa. The most characteristic images are observed after 14 days and 3 weeks of treatment (Fig. 2). In contrast, the appearance and the number of the Leydig cells do not seem to have been changed by the hypervitaminosis (fig. 3 and 4). The epididymis of the control group contained numerous spermatozoa (fig. 5). In the treated animals the spermatozoa were rare, the lumen contained numerous cells, mostly spermatophagous, but the epididymal epithelium remained prismatic and apparently normal (fig. 6). The seminal vesicles were well developed in all of the cases and there was never any change in their histological structure.

Comments. Hypervitaminosis E causes a significant reduction in the size of the testicles starting quickly after the commencement of the treatment. The atrophy is due to a halt in the spermatogenesis in most of the semen-producing tubes. After a month of treatment, however, the phenomena is less distinct and everything seems to indicate that the inhibiting effect of vitamin E was decreasing. The endocrine function of the testicle is not disturbed and the receptors of the male hormone did not change. The spermatozoa contained in the epididymis are much less numerous than normally and their disappearance is partially the result of the action of the spermatophages.

These results do not agree with any information taken from the medical literature and the images observed recall moreso the already conventional consequences of avitaminosis E. However, the effect observed seems to be transitional, since after a delay of about 3 weeks the spermatogenesis is restored. A similar fact was noted by Bastiani and Zatti [3] who, by means of heavy doses of vitamin E, caused functional and structural changes to occur in the corticosurrenal of the rat, followed by a gradual return to normal despite the continuation of the treatment.

It does not seem possible to us to interpret these initial observations and we propose to study the effect of hypervitaminosis E on the hypophysis of the adult animal and on the entire genital tract of the hamster during its evolution.

In summary, hypervitaminosis E causes rapidly in the hamster a significant testicular atrophy. The spermatogenesis is greatly disturbed but in a transitional manner. There is no change in the endocrine function of the testicle.

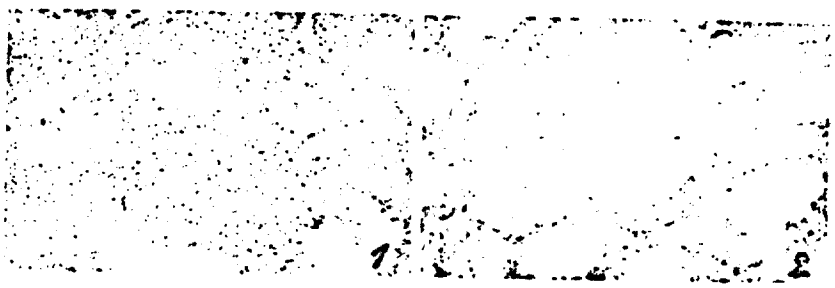
(Laboratory of Histiology and Embryology: Director M. L. Dumont, School of Medicine of Lyon France).

table key:

1= daily injections
2= duration of the treatment
3 = number of animals
4= weight of the two testicles at the time the animal was killed
5= jours = days
6 75 mg of vitamin E in solution in 0.75 cm^3 of oil
7= 0.75 cm^3 of neutral olive oil

Injections quotidiennes	Durée du traitement	Nombre d'animaux	Poids des 2 testicules au moment du sacrifice
75 mg de vitamine E en solution dans 0,75 cm³ d'huile	8 jours	2	1,2 g
			1,4 g
	14 jours	3	0,7 g
			1 g
			0,8 g
	21 jours	3	0,9 g
			0,6 g
			0,4 g
	30 jours	3	1 g
			0,6 g
			0,3 g
0,75 cm³ d'huile d'olive neutre	14 jours	2	2,5 g
			2,8 g
	30 jours	2	1,9 g
			2,4 g

Figure 1. Testicle of the control hamster: normal spermatogenesis.
Figure 2. Testicle after 14 days of treatment: spermatogenesis disturbed, absence of spermatozoa.
Figure 3. Leydig cells in the control testicle.
Figure 4. Normal Leydig cells after 14 days of treatment.
Fig. 5. Epididymis of the control hamster.
Figure 6. Epididymis after 14 days of treatment: the spermatozoa are rare, the sphermatophages numerous.



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THE INFLUENCE OF α-TOCOPHEROL ON THE URINARY ELIMINATION OF ANEURINE

L. D'Agostino

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Bari section -- Meeting of February 9, 1952

Several years ago we initiated the study of the relationships between vitamin E and other vitamins.

We have already reported on the modifications in urinary elimination of vitamin C and in the blood concentration of aneurine determined by the administration of α-tocopherol (1, 2).

Various clinico-therapeutical and experimental reliefs lead us to continue to consider as very probable the existence of relationships between thiamine and tocopherol. Such relationships were foreseen as early as 1938 by Bomskov (3), who succeeded in in avoiding the syndrome of deficiency in vitamin E in baby rats born of rats with vitamin E deficiency by administering beer yeast. Other researchers also report success in obtaining a diminution, or a complete disappearance, of the muscular manifestations of vitamin E deficiency, with the administration of thiamine (4. 5); however, this result was not confirmed by others (6).

Recently there has appeared a work by Bottiglioni (7), in which the author reports that he has verified an increase in the percentual urinary elimination of aneurine in the hours immediately follwing the administration of a dose of α-tocopherol.

Our preceding research documents a decrease of the blood value of aneurine liberated and combined during the treatment with vitamin E. (1)

The present research represents a further contribution to the study of the relationships between vitamin E and vitamin B_1 and has also the purpose of clarifying and better interpreting the mechanism of action of vitamin E.

In this note we report the results of 3 groups of experiments:

1) in a first group, we traced the curve of percentual elimination of aneurine in the 8 hours following the administration of a single dose of α-tocopherol, varying from 600 to 150 mg.

2) in the second group we dosed the daily urinary elimination of aneurine, first for 2 days without any treatment, and afterwards for 4 days with a daily administration of 500 mg of α-tocopherol to each of the subjects of the experiment.

3) in the third group we dosed the daily elimination of vitamin B_1, on the first 2 days without any treatment, then, for 2 more days, with the oral administration of 1.5 mg aneurine per

day, and finally, for 2 more days with the administration of 1.5 mg aneurine and 500 mg α-tocopherol.

In this manner we sought to establish the most adequate experimental conditions for avoiding the fact that possible reductions in the elimination of aneurine might be attributable to the exhaustion of the tissue vitamin reserve.

The experiments were conducted on subjects of a young age in good general condition, apparently exempt of metabolical alterations. The method of dosage of vitamin B_1: Ritsert (8).

TABLE I — Urinary elimination of aneurine in the various hourly fractions before and after α-tocopherol, in γ%

Casi N.	α-tocoferolo mg	Prima	Dopo 2 h	Dopo 5 h	Dopo 8 h
1	600	60	15	40	35
2	500	60	10	17,5	14
3	150	35,7	10	17,5	14
4	250	32	24	9	15

TABLE II — Daily urinary elimination of aneurine, in γ

Casi N.	α-tocoferolo mg	Prima		Dopo			
		1° g	2° g	1° g	2° g	3° g	4° g
1	500	168	172	140	120	98	105
2	500	280	266	190	165	120	135
3	500	256	268	210	180	146	154
4	500		757	760	270	190	238
5	500		353	402	205	117	139
6	500		412	149	131	267	115
7	500		420	144	51	243	160
8	500		807	830	337	152	116
9	500		1528	1443	1320	365	705

TABLE III — Daily elimination of aneurine, in γ

Casi N.	Prima		Vitamina B_1 mg 1,5		Vitamina B_1 mg 1,5 Vitamina E mg 500	
	1° g	2° g	3° g	4° g	5° g	6° g
1	480	360	600	500	360	300
2	410	404	480	400	200	220
3	410	220	300	400	240	192
4	380	420	472	320	280	240
5	280	292	260	340	100	122
6	360	388	400	440	270	196

Prima = before Dopo= after

The results reported in the tables reveal a decrease in the elimination of aneurine in the urinary fractions following the administration of a dose of α-tocopherol.

The quota of daily elimination of thiamine decreases during the administration of α-tocopherol.

The combined treatment with vitamin B_1 and vitamin E confirms these data, revealing again a decreased elimination of B_1 after tocopherolic treatment, always less than the quota of aneurine eliminated before the treatment in normal basal conditions.

We will return to the argument so that we can make the interpretation of these results clearer in the light of collateral research still under way. Nonetheless, the decreased urinary elimination of aneurine, and the already demonstrated decreased blood concentration during treatment with vitamin E, lead us to believe that this vitamin probably acts to limit the mobilization of vitamin B_1 from the tissues. In such a manner the synergism between vitamin B_1 and vitamin E verified by some authors and, on the other hand,, the increased requirement of vitamin B_1 in cases of vitamin E deficiency might be explained.

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* With the technical cooperation of Miss J. Begule, assistant biologist at the CNRS.

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** This product (acetate of bl. alpha-tocopherol in oily solution) was offered to us free of charge by the Byla Laboratories.

Current Science
14: 307-308, 1945

THE SPARING ACTION OF α-TOCOPHEROL ON CAROTENE

Moore[1] first observed that ingestion of extra vitamin E increased the storage of vitamin A in the liver of rats and lengthened the time taken to deplete them of vitamin A. This work was extended by Hickman and co-workers[2] who showed that vitamin E enhanced the growth-promoting activity of vitamin A and more markedly of carotene, the action being attributed to the antioxidative action of vitamin E. Guggenheim[3] has confirmed the results using a modified method for the bio-assay. Recently, however, Gridgeman[4] reported that workers in his and another laboratory failed to observe any synergism between vitamin A and carotene on the one hand and vitamin E on the other. Though he did not doubt the operation of the synergism, he suggested that it may not manifest under certain conditions (private communication). During the study of factors responsible for the variation in response to carotene when dissolved in different oils, one of us (S.D.R.) obtained evidence of the synergism.[5] The results have now been confirmed on more extended levels of dosage of vitamin E.

A number of vitamin A-deficient rats were each given 0·8 μg. of β-carotene six days in the week and assembled into five groups of six rats, one group receiving no α-tocopherol and the others increasing quantities of it as follows: 0·03, 0·12, 0·50 and 2·0 mg. immediately after the carotene. The vitamins were dissolved in cocoanut oil free from carotene and vitamin E, the total oil intake being 100 mg. per rat at a time. The experimental diet consisted of 68 per cent. sucrose, 18 per cent. extracted casein, 10 per cent. brewer's yeast and 4 per cent. Osborne-Mendel salt mixture fortified with 1 mg. thiamine hydrochloride and 1 mg. of riboflavin per 1 kg. of diet and supplemented with 1 μg. of calciferol per week. The B vitamins were added since Hickman has pointed out the need for their adequate intake. The weekly gain or loss in weight was noted; the results are given in the accompanying table.

Effect of varying supplements of tocopherol on growth induced by 0·8 μg. carotene

Group	Tocopherol supplement mg.	Gain in weight in g. at the end of 1 week	2 weeks	3 weeks	4 weeks	5 weeks
1	Nil	1	−4	−7	−7(5)	All dead
2	0·03	0	2	5	4 (1)	2 (3)
3	0·12	0	4	5	5 (2)	7 (3)
4	0·50	6	8	10	11	14
5	2·0	3	3	4	8	12

*The figures in parenthesis indicate the number of rats that died during the experiment.

A dosage of carotene which was insufficient by itself to maintain life in rats became sufficient and promoted normal growth when administered together with tocopherol. The optimum tocopherol supplement appears to be the same as that observed by Hickman, *viz.*, 0·5 mg. per rat, though the diet used here was lower in fat.

Our grateful thanks are due to Prof. V. Subrahmanyan for his kind interest in the work.

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October 19, 1945.

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THE PROBLEM OF THE TUMOR PRODUCING EFFECT OF WHEAT GERM OIL

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Recently, a number of publications appeared concerning the alleged tumor forming effect of wheat germ oil.

Wheat germ oil favored the growth of inoculated adenocarcinoma of male mice, the appearance of experimental tar carcinoma (Severi /1/), produced malignant transplantable fusiform cell sarcoma in the adbominal cavity of rats (Dorrance-Ciccone /2/, Rowntree-Lansburry-Steinberg /3/.) The feeding of residues of the ether extract of wheat germs (Rowntree-Steinberg-Dorrance-Ciccone /4/ led in rats to similar results. Ether extracts of raw wheat germ oil (Rowntree-Steinberg-Brown /5/) caused the appearance of transplantable tumors in the abdominal cavity of rats. It appears, however, that the vitamin E contained in wheat germ and wheat germ oil has no part in the tumor forming effect. The authors emphasize that refined wheat germ oil and vitamin E concentrates, linseed oil, cod liver oid and oleic acid do not have carcinogenic properties.

Vitamin E deficiencies do not inhibit the growth of rat carcinoma (Zagami /6/. Severi /4/) and did not reduce the number of spontaneous mamma carcinoma in mice (Carruthers /7/).

The addition of wheat germ oil to the food of mice treated with methylcholanthren (Carruthers /7/) and with benzpyrene (Haddow-Russel /8/) did not enhance the growth of tumors. Vitamin E containing plasma affected the growth of fibroblasts in vitro negligibly (Gordonoff-Ludwig /9/). An inhibiting effect of wheat germ oil on the development of experimental tar carcinoma has been described (Davidson /10.)/

As is seen, the views of the authors cited above are widely different. According to some, wheat germ oil is incapable of producing tumors or to accelerate their growth, while others demonstrate the tumor forming effect of wheat germ oil, leaving the problem whether the effect is due to the vitamin E contained in the oil or to some other unknown component, undecided. Finally, an inhibiting effect of wheat germ oil on tar carcinoma has been described.

The synthesis of vitamin E (alpha-tocopherol) by Karrer /11/ (1938) made it possible to perform experiments with large doses of the pure vitamin (dl-alpha-tocopherol) or its acetate on mice and rats, which permit the formulation of an opinion concerning the results obtained by the authors cited above.

It was first attempted to obtain a carcinogenic effect by smearing the skin on the back of mice with a 10% oily solution of the synthetic vitamin E. Then, the development of precancerous and carcinomatous skin phenomena produced on mice with benzopyrene was observed and an attempt was made to affect their development by the oral administration of vitamin E. Further, these experiments were performed on rats in which sarcoma were produced through the subcutaneous injection of benzopyrene. Finally, substantial amounts of synthetic vitamin E were given os to rats in order to determine whether tumors are produced in this manner.

I. Treatment of the Skin of Mice with dl-alpha-tocopherol. Comparison with Benzopyrene

The experiments were performed on 20 adult male mice, of which 10 were smeared 3 x per week with a 10% dl-alpha-tocopherol solution in sesame oil on their depilated backs, while the remaining 10 were treated in a similar manner with a 1% benzopyrene solution in benzene.

On the animals treated with vit. E, the hair cover reappeared as early as 8-10 days after the treatment (as on the untreated control animals). Initially, thin lanugo hairs appeared which later developed into normal hairs. The skin remained supple and smooth without new formations of any kind (experimental period 8 weeks).

The mice treated with benzopyrene were incapable of replacing their hair cover. They reacted after 2-3 weeks with a thickening of the skin on their backs and incipient callosity. Some of the animals later developed warts and papilloma.

II. Treatment of Precancerous and Carcinomatous Skin Phenomena on Mice With dl-alpha-tocopherol

Fully grown female mice were used as experimental animals, on which the smearing of the skin of their backs (3 x per week) with a 1% benzopyrene solution in benzene lead to loss of hair, thickening of the epithelium, growth of warts, papilloma and carcinoma. Of this animal material, two groups of 10 mice each were formed, of which two of each were afflicted with skin carcinoma on the back, two with warts or papilloma and the rest with skin thickening and callouses. All of the animals were further treated with benzopyrene. In addition, one half of them received a daily dose of 10 mg dl-alpha-tocopherol per os.

The warts, tumors, etc., were measured twice weekly (length, width, height) and new formations entered in a diagram (outline of mouse). During this time, the surface of the ulcers of the animals treated with vit. E increased by 45-80%, that of the control animals by 50-130%. With the considerable variation observed in the growth of the tumors, the difference is meaningless.

All of the animals produced sections exhibiting infiltrating growth of the carcinoma in the subcutis and muscles; pulmonary metastasis was observed in one case only.

The rest of the mice was observed for a month. During this period of time warts and papilloma increased in size. A few regressed; this had been observed on an earlier occasion also. The thickening of the skin was not reduced in any of the cases, new warts formed, callouses spread.

No appreciable difference was found between untreated control animals and the experimental animals.

III. Treatment of Fusiform Cell Sarcoma of Rats with dl-alpha-Tocopherol

Twelve male adult albino rats, injected 4 months earlier with 1 ccm of a 1% oily benzopyrene subcutaneously in the back, were used as experimental animals.

At the start of sarcoma formation, 6 pairs were selected, taking care that the sarcoma should resemble each other as closely as possible with respect to shape and size. One of the animals of each pair was treated with vit. E while the other served as control and remained untreated.

The animals treated with vit. E received 3 times weekly 0.2 ccm of a 10% solution of dl-alpha-tocopherol in sesame oil (=60 mg dl-alpha-tocopher per week) per os. During the entire experimental period the animals were fed a conventional mixture of feed. They were examined twice weekly, the size of the tumor was measured with a compass and its volume determined (length x width x height). Compact, approximately cubic new formations yielded the most accurate values. In cases of irregular formatins, several transverse measurements were taken; in partitioned tumors, individual formations were measured separately.

Following exitus (spontaneous or by narcosis), all of the animals were dissected and the volume of the tumors determined by submersion in a graduate column filled with physiological salt solution. Histological examination showed fusiform cell sarcoma without expection.

Table 1 shows the volume of tumors of animals treated with vit. E and of untreated rats after two weeks and at the termination of the experiment. Based on these data, the growth of tumors was calculated after two weeks and after the end of the experiment. Since some of the animals perished prematurely, the experimental periods are unequal for different pairs of rats. The shortest period was 14 days (Pair IV), the longest 39 days (Pair V).

a) Growth of Sarcoma in Rats without Vitamin Additions

As is seen, the tumors of the individual control animals varied strongly at the start. The smallest sarcoma, with 1.8 cm^3, is roughly

the size of a cherry, while the largest (37 cm^3) corresponds in size approximately to a plum. The dimensional ratio thus was about 1:20. At the end of the experiment, on the other hand, the smallest tumor was 61 and the largest 233 cm^3. The dimensional ratio thus is only 1:4.

The table shows that the growth of individual tumors varies strongly. During the experiment, some of the tumors increased 2-6 times in size, while others became 45-49 times larger. These extreme differences must be considered in the evaluation of a potential effect of vit. E on the growth of tumors.

b) Growth of Sarcoma in Rats Treated with dl-alpha-Tocopherol.

The difference between the initial and final volume of tumors in rats treated with vit. E is just as large as with the control animals. In the case of the experimental animals, the volume of individual tumors is closer than at the beginning. At the start the tumors differ in ratio of 1:44, at the end as 1:4.2. Growth varies from case to case. In Animal 89 the tumor grew 15 times, in Animal 86, 24 times.

Table I. Comparision of the Growth of Fusiform Cell Sarcoma in 12 Rats Produced by the Subcutaneous Injection of Benzopyrene. Six Rats Received 20 mg Vitamin E Daily Synth. per os (During the entire experimental period).

Key: 1. Experimenta; pair; 2. No. of experimental animals (with vit. E); 3. No. of control animal (without vit. E); 4. Length of period in days; 5. Volume of sarcoma (cm^3); 6. at start; 7. after 14 days; 8. at termination; 9. increase in tumor volume; 10. increase in tumor volume (times initial volume); 11. total; 12. experimental animals; 13. control animals; 14. average value per animal.

Tabelle I.

Vergleich des Wachstums von durch subcutane Einspritzung von Benzpyren erzeugten Spindelzellensarkomen an 12 Ratten, wovon sechs täglich 20 mg Vitamin E synth. per os (während der ganzen Versuchsdauer) erhielten.

Versuchspaar (1)	Versuchs-Tiere Nr. (mit Vit. E) (2)	Kontroll-Tiere Nr. (ohne Vit. E) (3)	Dauer in Tagen (4)	Volumen der Sarkome (cm^3) (5) zu Beginn (6)	nach 14 Tagen (7)	Ende (9)	Zunahme des Tumorvolumens (cm^3) (9) nach 14 Tagen (7)	Ende (8)	Tumorzunahme (x Anfangsvolumen) (10) nach 14 Tagen	Ende
I	89	88	24	4,72	48,36	79,20	43,63	74,50	9,28 x	15,86 x
			24	24,64	77,44	176,25	52,79	151 60	2,19 x	6,31 x
II	0	91	24	5,81	37,69	111,13	31,88	105,31	5,49 x	18,15 x
			24	3,34	16,84	61,78	13,50	58,44	4,09 x	17,70 x
III	86	84	24	8,22	86,98	206,38	78,75	198,15	9,60 x	24,16 x
			24	10,35	132,46	232,74	122,11	222,38	11,85 x	21,59 x
IV	85	82	14	44,19	141,69	141,69	97,50	97,50	2,21 x	2,21 x
			14	37,44	119,93	119,93	82,49	82,49	2,22 x	2,22 x
V	K	K St	39	1,84	25,72	164,64	23,88	162,79	13,26 x	90,43 x
			39	4,62	46,75	233,85	42,13	229,23	9,15 x	49,83 x
VI	KR	94	31	7,12	64,17	339,76	57,05	332,64	8,03 x	46,85 x
			31	1,82	9,43	84,56	7,61	82,74	4,22 x	45,96 x
Total: (11)						(12) Versuchstiere	332,69	970,95	47,87 x	197,66 x
						(13) Kontrolltiere	320,63	826,88	33,72 x	143,61 x
(14) Durchschnittswert pro Tier						Versuchstiere	55,45	161,82	7,97 x	32,94 x
						Kontrolltiere	53,44	137,81	5,62 x	23,03 x

In spite of considerable differences in the size of tumors in untreated control animals and experimental animals treated with vit. E, both at the beginning and the end of the experiment, the average values of the two groups of animals exhibit a remarkable agreement. The tumors of the control animals increased on the average 23 times, those of the experiment animals, treated with vot. E, 32 times. The average final volume of the two groups differed only by approv. 24%, a negligible difference. The experiment therefore did not succeed in stimulating the experimental benzopyrene sarcoma of rats to increased growth, the plentiful administration of vitamin E notwithstanding.

IV. Chronic Administration of Vitamin E to Normal Female Rats

The experimental animals were 12 normal white rats weighing 90-110 g. During a period of 49 days, daily 20 mg dl-alpha-tocopherol (a total of 980 mg per day) were given orally. The 12 rats increased normally in weight. They were paired, became pregnant and produced normal offspring. After 4 months, they were killed and dissected. All were healthy. Not one exhibited tumors.

The experiments show unambigously that vit. E (synthetic dl-alpha-tocopherol of its acetate) have no carcinogenic properties. Following the smearing of the depilated skin of mice with a 10% dl-alpha-tocopherol solution, hair is replaced (as with normal, untreated control animals) and no new formations appear. Oral administration of vit. E in high doses (10 mg daily for 20 days = 200 mg) has no effect on precancerous skin symptoms of mice produced by benzopyrene. The benzopyrene carcinoma of mice and benzopyrene sarcoma of rats are equally unaffected by treatment with vit. E.

Oral administration of vit. E to normal rats (20 mg daily for 49 days = 980 mg) produced no tumor in any of the cases.

Based on these experiments, there is no doubt that tumors appearing following the administration of raw wheat germ oil, as described in the literature, cannot be attributed to the vit. E. contained in wheat germ oil.

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THE PHARMACOLOGY OF VITAMIN E

(tolerance to synthetic DI-α tocopherol and its acetate)*

by V. Demole

received Feb. 25, 1939

(from the Pharmacology Laboratory of the F. Hoffmann-La Roche Co. A. G., Basle).

Tocopherols are thick fluid oils which are insoluble in water but which, however, are easily soluble in most organic solvents.

The natural tocopherols (α,β,γ) were first isolated by Evans et al.[1] (1936) from various plant germ oils in small amounts so that an examination of the toxic effect was not possible. Only since the synthesis of vitamin E by Karrer[2] (1938) have we had enough amounts available to determine the tolerance through delivering a one-time dose or repeated daily doses from 10 to 30 days. A short report on these tests is given ahead. All of the tests were carried out both with the synthetic dl-α and with its acetate. For purposes of brevity we will use the term "vitamin E" for both preparations.

I. Delivery of a one-time dose

In the oral administration of pure vitamin E in the dosage of 50 g/kg mouse (therefore 1 gram for a mouse of 20 grams in amounts of 0.33 ccm in intervals of 4 hours)) an irregular decrease in weight (2-3 g) was observed which, however, only lasted 3 to 4 days and was probably due to loss of appetite.

The rat of 200 g body weight tolerated easily 1 g of pure vitamin E in oral administration under the conditions described for the mouse. This dose is 500 times greater than the dose of 2 mg antiabortive active to 80-100% on the E-avitaminotic rat. Per os, frogs, rabbits, cats, dogs and monkeys tolerated 0.2 g/kg of vitamin E without symptoms (10% solution in sesame oil). The same dose was also tolerated without symptoms in frogs, rats, rabbits and dogs when administered subcutaneously, intramuscularly, or interperitoneally. In these types of animals it caused no local stimulant effect, except in the case of intracutaneous injection of 0.1 ccm of the 10% solution in rabbit ear which resulted in a temporary tissue stimulation (hyperemia lasting about 24 hours). In the case of the mouse, however, a surface necrosis of the skin occurred after subcutaneous injection. The stimulating effect, however, is to be traced to the solvent since the injection of 0.2 ccm of pure

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vitamine E was tolerated without any symptoms.

II. Repetition of daily injections during 10 days

The administration of vitamin E on 10 consecutive days was well tolerated by the male rat in the dose of a total of 1 g /kg orally. The normal female rat, whether it be a virgin or pregnant, as well as the E-avitaminotic rat tolerated 0.5 g/kg orally without any symptoms. A dog and cat received orally a dose of 0.1 g/kg, a pidgeon orally 2.1 g/kg, a mouse subcutaneously and intraperitoneally 2.5 g/kg, orally 25 g/kg. All of these animals stayed healthy. Did not exhibit any changes at all.

A histological investigation was carried out on the heart, lungs, liver, kidneys and testicles of several rats, rabbits and cats which were used for these tests. Pathologic-anatomic changes could not be detected. The fertility of the rats remained the same. The pregnancy was neither shortened nor lengthened. The number and weight of offspring was normal, the growth accelerated. More will be reported on this point in a later publication. The milk secretion of the mother animal was plentiful. The young were regularly removed on the 24th day.

III. Daily Administration during 1-2 months

The vitamin E was administered for a period of two months orally to a group of 10 male and 10 female rats in a total dose of 4 g/kg. This dose was tolerated without any problem. The estrum cycle of the rat, the fertility, the duration of the pregnancy, the number of children and their weight, and the milk secretion of the mother animal were unchanged. In comparison with the control animals, the testicles of the normal rats treated with vitamin E showed no significant changes in weight. The spermatozoa continued to move. The vitamin E was also given in doses of a total of 0.21 g/kg to the dog and total of 0.24 g/kg to the cat in the course of one month. All of the animals remained completely healthy. No loss in weight was observed. Urine examinations of the dog with regard to albumen and sugar resulted negative.

Summary

Vitamin E (synthetic dl-α, and the acetate thereof did not seem to exert any toxic side-effects. The greatest dose administered to the mouse (50 g/kg orally) was tolerated well. No poisoning symptoms at all were observed in rats, dogs and cats which had been given several grams of vitamin E for 1 to 2 months. There were no peculiar findings in the kidney, intestinal functions, as well as in the neurological, muscular and sexual functions.

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UTILIZATION OF PURE α-, γ-, AND δ-TOCOPHEROLS BY LAYING HENS[1]

MEI YU DJU, MARY L. QUAIFE AND PHILIP L. HARRIS

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THE non-α-tocopherols (β-, γ-, and δ-) are considerably less potent than α-tocopherol in their ability to prevent sterility and muscle dystrophy although they do not differ greatly in chemical structure. It has been suggested that what potency they do have is due to conversion to α-tocopherol. To test this possibility, hens maintained on a vitamin E-deficient diet were fed supplements of pure natural γ- or δ-tocopherol and their eggs were assayed to show whether or not the α-tocopherol content was increased. Information was also obtained regarding the relative deposition of α-, γ- and δ-tocopherol in hen's eggs.

METHODS

Mature hens were kept in individual cages in air-conditioned rooms (75° F.; 50% R.H.) and fed a commercial laying mash until a regular egg production pattern was established. They were then transferred gradually over a period of 10 days to a vitamin E-deficient diet, similar to that of Dam (1), table 1.

On this diet hens showed markedly decreased egg production or stopped laying altogether. Supplements of the various pure, natural tocopherols[2] were given in capsules, usually 5 per week. The dosage level varied from 100 to 4000 mg/week, each level being fed for 2 to 4 weeks.

Eggs were collected and stored in the deep-freeze at −22°C. until time of analysis. They were analyzed for total and for γ-plus δ-tocopherols, α-tocopherol being calculated by difference. Tocopherol analyses were made on occasional samples of blood and feces.

The assay methods for total and for γ- plus δ-tocopherols in eggs consisted primarily of the steps described for analysis of foods for vitamin E (3). However, 2 extraction techniques were compared: *1*) extraction with a 1:1 mixture of chloroform and ethanol, followed by filtration, evaporation and re-extraction of the residue with chloroform, and *2*) extraction with a mixture of 50 per cent ethanol and petroleum ether. Both procedures extracted more carotene than could be conveniently decolorized in the semi-micro hydrogenator. Accordingly, weighed portions of the lipid extract from eggs were molecularly distilled, thus effecting a separation of part of the carotene from the tocopherols. The distillate containing the tocopherols was then hydrogenated and assayed photometrically for tocopherols.

Extraction according to the first procedure always gave more lipid and usually

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[2] Kindly supplied by the Organic Research Dept. of Distillation Products, Inc.

more tocopherol than did extraction according to method 2). The maximum difference was about 25 per cent. However, the proportion of γ- and δ- to total-tocopherols was the same with both techniques. Since only comparative tocopherol values in eggs were wanted, the simpler alcohol-petroleum ether extraction procedure was used.

In this, 8 gm. of well-mixed whole egg + 8 ml. of distilled water + 16 ml. of absolute ethanol + 40 ml. of purified Skellysolve B in a glass-stoppered cylinder were shaken for 10 minutes. Twenty milliliters of the supernatant were evaporated to dryness under nitrogen in the aluminum cup which lines the molecular still. The lipid residue was weighed, and then distilled, hydrogenated, and assayed by photometric methods for tocopherol content.

TABLE 1. COMPOSITION OF DIET NO. 300[1]

Ingredient	%
Casein, crude	15.0
Dried yeast	10.0
Corn starch	50.8
Gelatine	8.0
Gum arabic	4.0
Salt mixture[2]	7.0
L-cystine	0.1
Choline chloride	0.1
Vitamin K (*10 mg/kg.*)	—
Cod liver oil (Added fresh daily to mixture of dry ingredients)	5.0
	100.0

[1] The vitamin E-free diet assayed 1.67 mg/100 gm of total tocopherols and 0.19 mg/100 gm of γ- plus δ-tocopherols. However, these results were probably caused by a tocopherol-like substance contained in the yeast of the diet. Such a material has been studied. It is fat-soluble, non-saponifiable, Emmerie & Engel (2) positive before and after hydrogenation, and it couples with diazotized dianisidine. However, it shows no biopotency in rat anti-sterility assays. Hence, the diet can be considered virtually vitamin E-free.

[2] Salt mixture: U.S.P. No. 2 salt mixture, 348 gm., plus manganese sulfate, 2 gm.

All egg assays were done at least in duplicate and usually in quadruplicate. Replicate assays showed excellent agreement.

RESULTS

In a preliminary experiment, 3 hens were used. These produced eggs with total tocopherol content of 2.5 to 4 mg/100 gm. of whole egg. This range of values decreased to 0.4 to 1.0 mg. after 2 weeks of depletion during which the vitamin E-free diet No. 300 was fed. Weekly supplementation of these hens with 100 mg. of d,α-tocopherol caused the total tocopherol content of eggs to increase to 4 to 15 mg/100 gm. However, γ-tocopherol supplementation at the same level resulted in no increase in egg tocopherol concentration. Higher levels of γ-tocopherol supplementation were necessary to produce eggs with enhanced total tocopherol content. For example, a hen, which laid eggs with 9.00 mg. of tocopherol/100 gm. after supple-

mentation with 100 mg/week of α-tocopherol, laid eggs with only 2.5 mg. of tocopherol/100 gm. when fed 500 mg. of γ-tocopherol per week.

The extra tocopherol, deposited in eggs as a result of high-level γ-tocopherol supplement to the hens' diet, appeared to be chiefly γ-tocopherol.

To confirm and extend these observations a second experiment was made. Laying hens, weighing between 3.5 and 5 lb., were maintained on laying mash for one week and then shifted gradually (10% each day) to the vitamin E-free diet No. 300. During the first week, average values were 0.88 to 1.53 mg. of total tocopherols/100 gm. and 0.12 to 0.30 mg. of γ- plus δ-tocopherols, respectively. Thus, eggs produced by hens on a presumably normal laying ration contained tocopherols, approximately 85 per cent of which was α-tocopherol.

During the conversion from commercial mash to vitamin E-free diet there was little change in the tocopherol content of the eggs. However, after the hens had been on diet No. 300 for a week, the tocopherol content of all the eggs was definitely lower, e.g. 0.16 to 0.64 mg/100 gm., and practically 100 per cent of this vitamin E was α-tocopherol.

After several weeks on the vitamin E-free diet, the hens showed changes in egg production. For the first 5 weeks they laid eggs at a constant and characteristic rate (4 to 6/week). At 8 and 9 weeks the rate dropped to 1 or 0/week for each of the hens. At this time the hens had blood vitamin-E levels of less than 0.1 mg/100 ml.

Supplements of the various pure tocopherols were started at the eighth or ninth week. Egg production increased immediately, sometimes to pre-depletion levels, although the rate fluctuated depending upon the level of α-tocopherol supplementation.

No evidence was found in this experiment to show that there is conversion of non-α- to α-tocopherol. Hens fed δ- or γ-tocopherol laid eggs with increasing proportion of non-α-tocopherol, i.e. up to 90% of total, as the level of supplementation was increased.

The prompt and steep increase in amount of tocopherol deposited in eggs of hens which received α-tocopherol is in marked contrast to the slow and slight increase of tocopherol in eggs laid by hens on γ- or δ-tocopherol supplementation. This is evident from figure 1 in which concentration of total tocopherols in eggs as a function of log dose (weekly supplement) is plotted. The values for egg tocopherol concentration are those found in the second week of supplementation of hens at the given level; limited supplies of the pure tocopherols precluded longer supplementation at every level. However, change in egg tocopherol concentration between the second and third weeks was slight, compared to that between the first and second week of hens on supplement. Tocopherol concentration in eggs increased proportionally to the log-dose (fig. 1). The slopes of the curves are 31, 7, and 3 for α-, γ-, and δ-tocopherols, respectively. Consequently, γ-tocopherol was only about one fourth as effective as α-tocopherol and δ-tocopherol only about one tenth as effective as α-tocopherol in these experiments.

This relation between amounts of the pure tocopherols laid down in eggs is consistent with the relative physiological activity of the tocopherols in relieving a variety of deficiency syndromes in several species. It is similar to data on relative

amounts of deposition of various tocopherols in body tissues of rats (4). Studies in our laboratory have shown that cows fed equal amounts of α- or of γ- plus δ-tocopherols secrete proportionately much more of the former than the latter in milk (5). Assays of human tissues and calculations of dietary intake of the various forms of tocopherol suggest a similar relation for deposition of ingested tocopherol in the body tissues of humans (6).

The efficiency of transfer of tocopherols to eggs reached a maximum in these experiments at the lowest levels fed (100 to 200 mg/week). This efficiency is expressed as follows:

$$\% \text{ efficiency} = 100 \times \frac{\text{total tocopherol in eggs laid/week}}{\text{amount of tocopherol fed/week}}$$

Typical efficiency values for α-, γ-, and δ-tocopherol-fed hens were, respectively, 16.5, 2.9, and 1.4 per cent.

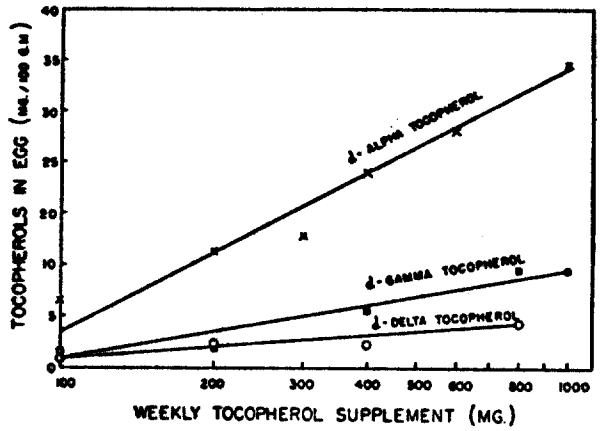

Fig. 1. TOCOPHEROL CONCENTRATION in hens' eggs (2nd week of pure tocopherol supplement).

Preliminary data regarding the vitamin-E requirement of laying hens may be drawn from these experiments. The laying mash contained, by chemical assay, 6.29 mg. total tocopherols/100 gm. Because of interfering materials, non-α-tocopherols could not be assayed chemically. They may be assumed to comprise one half of the total, since bioassays of a wide variety of grains in our laboratory have shown that about 50 per cent of total tocopherols are non-α-tocopherols. If a hen consumed 120 gm/day of mash, about 3 mg. of α-tocopherol/day would be derived from the diet. On the average, each hen laid 5 eggs/week, each weighing about 55 gm., or about 275 gm. of egg/week. Eggs laid during this period averaged about 1.2 mg. of α-tocopherol/100 gm. Thus, of 21 mg. of α-tocopherol which the hens received from the diet, they put out (275 × 1.2/100) 3.30 mg. in eggs. The efficiency of transfer (3.30/21 × 100 = 15.7%) is about equal to that calculated for α-tocopherol-fed hens at the lower levels of supplementation in these experiments.

Thus, a daily intake of 3 mg. α-tocopherol by the hen permitted egg production with higher than minimal α-tocopherol content, normal metabolic processes, and

probably some storage of α-tocopherol since 8 to 9 weeks were required to deplete these hens of stored tocopherols. Assuming a weekly output of 5 eggs, a minimum concentration of 0.5 mg. tocopherol/100 gm. of egg, and 55 gm. the average fresh weight of eggs, then $\frac{5 \times 0.5 \times 55}{100} = 1.37$ mg. of tocopherol, the minimal weekly output of tocopherol. The minimal weekly intake would then be (100%/16% × 1.37 mg.) 8.6 mg. of α-tocopherol or about 1.2 mg/day which might be considered the minimum requirement.

Blood levels of vitamin E were determined on several of the hens on various tocopherol supplements. After one week of supplementation with 1600 and 2000 mg. of α-tocopherol, respectively, 2 hens had plasma vitamin-E levels of 20.0 and 20.1 mg/100 ml. In contrast, a hen on 1000 mg/week of γ-tocopherol had 2.1 mg. per cent tocopherols in blood, and a hen which received 800 mg/week of γ-tocopherol had 1.35 mg. per cent tocopherols. It is evident that blood tocopherol levels of hens, like the amounts in their eggs, are much higher following α-tocopherol than γ- or δ-tocopherol supplementation.

Occasional fecal samples from the hens were assayed for total tocopherols and for individual tocopherols by both dianisidine-coupling (7) and nitroso-assay methods (8). In all cases the preponderant form of tocopherol excreted was the same as that fed. No conversion of one form of tocopherol to another was indicated. One of the hens which was fed one gm/day of α-tocopherol excreted 770 mg. in a 24-hour fecal sample.

SUMMARY

Laying hens, maintained on a vitamin E-free diet, were given supplements of pure α-, γ-, or δ-tocopherols at levels of 100 up to 4000 mg/week. Their eggs were assayed for total and for γ- + δ-tocopherol content. α-Tocopherol content was estimated by difference. No evidence of conversion of γ- or δ-tocopherol to α-tocopherol by the hen was found. The tocopherol concentration of the eggs varied linearly with the log-dose. The α-tocopherol induced the greatest deposition; γ-tocopherol was one fourth and δ-tocopherol was one tenth as effective as α-tocopherol. From these preliminary data, it is calculated that the laying hen requires a minimum of about 1.2 mg/day of d,α-tocopherol. Large amounts of unchanged tocopherol were excreted in the feces of hens on high level supplements of various pure tocopherols. Blood-tocopherol levels of hens on α-tocopherol supplement were several-fold greater than those of hens receiving similar amounts of γ- or δ-tocopherols.

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Minimal Doses of Alpha- Tocopherol Acetate Necessary to Prevent Sterelity in the Male White Rat, by:

Drs. Adolfo Escudero and Maria Luisa Herraiz

Motive for the present work:

In a previous work on "The Effect of a deficient and excessive dietary regime in fat (Escudero P. and others, 1940) obtained results that led them to suspect that diets which are very deficient in fat aggrevate the symptoms due to lack of vitamin E. Finally, comparing these extremes, it was considered necessary to determine the minimal dose of vitamin E necessary for the male white rat. For these reasons the present investigation was begun in June of 1939.

Anticedent:

Until now we had not been able to encounter more than two works that referred to this theme. One was undertaken by H. W. Julius and C. H. Engel and was referred to in chemical abstracts in 1940. The other was done by K. E. Mason in 1940. Of the two works, we were able to obtain that of Mason. This publication came into our hands in January of 1941 when our work had already been concluded under circumstances that prevented us from taking advantage of suggestions contained in it.

Masons work allowed us the satisfaction of comparing our work with his. Mason considered the minimum dose of vitamin E necessary for the male white rat to be between 37 and 70 micrograms and our work showed it to be equal to 30.

It became necessary to explain the differences encountered in the two works.

Experimental Part:

Diet Utilized: The diet utilized consisted of the following elements:

Caseen	180 g
Wheat starch	735 g
Mixture of salts	40 g
Beer Yeast	35 g
Cod liver oil (containing 7.200 U. I. of vitamin A and 1.000 U. I. of vitamin D	4 ml.

This diet was administered ad-libitum.

Animals Utilized: 21 day old males were used. These animals behaved normally toward the females as was our "general belief". During the first two weeks of the period of location the animals received the same food as during pregnancy. When the brood reached the age of 14 days, the mothers were administered a diet lacking in vitamin E as was mentioned before with the objective of depriving the brood of vitamin E at the same time as the mothers. At the end of 21 days of age the animals were separated from the mother and were given varying doses of vitamin E. The rats were distributed into groups. Each group consisted of as large a number of brothers as possible as the other rats with the objective of avoiding the use of animals with varying reserves of vitamin E which could produce errors.

Established Animal Groups: The animals used in this experiment were distributed into eight groups. In addition to the basic diet without Vitamin E, the groups received the following supplements:

Table 1 Supplements given the experimental animals.

CUADRO 1

Suplementos suministrados a los animales en la experiencia

(1) Lote	(2) Suplemento
1	0,6 mg de acetato de alfa tocoferol (3)
2	0,3 " " " " " "
3	0,06 " " " " " "
4	0,03 " " " " " "
5	0,015 " " " " " "
6	0,006 " " " " " "
7	10 mg diarios de insaponificable de germen de trigo (4)
8	Ningún suplemento (5)

Key: 1. Group; 2. Supplement; 3. of alpha tocopherol acetate; 4. daily mg of emulsified wheat germ; 5. no supplements.

The last two groups served as a test group for the first six. A synthetic Vitamin E supplement was administered to the different groups which was calculated to be in agreement with the following precedents encountered in the literature. Bacharach, in 1938, established the theory that a single dose is sufficient to prevent reabsorption in the brood of a female lacking vitamin E, a dose equal to $\frac{6N}{100}$ administered daily from birth would be sufficient

General Method of the Experiment: The experiment lasted 25 weeks. During the first period the animals were weighed weekly. The weight increment for all groups was approximately 300 grams. The smallest difference obtained in favor of the groups which received vitamin E was not statistically significant. No untouched symptoms were observed. The animals ate daily and without exception ate the Vitamin E supplement offered them. The animals were mated 22 weeks later after the beginning of the experiment. Two weeks later the animals were sacrificed.

Results Obtained Through the Medium of Fecundity Testing: At the end of 22 weeks from the beginning of the experiment, the animals were placed together with normal females for 7 days (90-100% fertile). At the end of this period, the males and females were separated. The females were kept for 10 more days on the food of the general nursing rats rich in vitamin E. The existance of pregnancy and the number of animals contained in the uterus was determined by autopsy.

The results obtained are detailed in the following:

Table II. Results of Fecundation Test.

Lote (1)	Suplemento (2)	Número de hembras cruzadas (3)	Número de hembras fecundadas (4)	Número de animales contenidos en el útero (5)		Porcentaje de fecundación (7)
				Total	Promedio por hembra (6)	
1	0,6 Acetato de alfa tocoferol (8)	6	4	49	12,2	66
2	0,3	8	5	57	11,4	62
3	0,06	8	6	81	13,5	75
4	0,03	6	6	66	11,0	100
5	0,01	8	2	18	9,0	25
6	0,006	6	0	0	0	0
7	Insaponificable de aceite de germen de trigo (9)	10	9	127	14,1	90
8	Ninguno (10)	15	0	0	0	0

Key: 1. Group; 2. Supplement; 3. No. of bred females; 4. No. of fecund females; 5. No. of animals in uterus; 6. average per female; 7. % of fecundation; 8. tocopherol acetate; 9. oil emulsion of wheat germ; 10. No. supplement.

The results of the preceeding table show that 30 gammas of alpha-tocopherol acetate administered in minimal daily doses for 22 weeks prevented sterility in 100 % of the animals used.

Results obtained through the medium of weighing testacles:

It is known that a lack of vitamin E produces much atrophy of the testacles. Based on this fact, it was decided to determine the minimal dose of alpha-tocopherol acetate necessary to prevent atrophy of the testacles in the male white rat. To determine the weight of the testacles the animals were sacrificed two weeks after terminating of breeding. The testacles were immediately taken away and weights of the dried testacles were obtained. They were dried at 80°C. The results were as follows:

Table 3, Results obtained in weighing testacles.

Lote (1)	Suplemento (2)	Testículo húmedo (promedio en gramos) (3)	Testículo seco (promedio en gramos) (4)	Humedad porcentaje (5)
1	0,6 mg de acetato de alfa tocoferol (6)	1,626	0,229	85,8
2	0,3 " " " "	1,654	0,245	85,1
3	0,06 " " " "	1,649	0,237	85,7
4	0,03 " " " "	1,727	0,251	85,4
5	0,015 " " " "	1,257	0,185	85,4
6	0,006 " " " "	1,693	0,068	90,1
7	Insaponificable de aceite de germen de trigo, 10 mg (7)	1,702	0,242	85,6
8	Ninguno (8)	0,710	0,084	88,1

Key: 1. Group; 2. Supplement; 3. wet average in grams; 4. dry average in grams; 5. wer%; 6. mg of tocopherol acetate; 7. emulsion of oil and wheat; 8. no supplement.

From the aforementioned data it can be seen that:

1. The minimal dose of alpha-tocopherol acetate needed to prevent atrophy is equal to 30 gammas when administered daily for 26 weeks.

This dose is the same as the minimum required to prevent sterility in the animals in the experiment.

Results obtained by studying the histology of the testacles:

The histological study of the testacles was realized in the animals of groups 1, 4, 5, 5, 7 and 8. The following results were obtained.

Table IV, Results of the Histological Study of the Testacles.

Lote (1)	Suplemento (2)	Espermatozoides histológicamente normales conservados (en gramos) (3)
1	0,6 mg de acetato de alfa tocoferol (4)	El 100 % de los animales estudiados (5)
4	0,3 „ „ „ „	El 100 % de los animales estudiados
5	0,015 „ de vitamina E	El 50 % de los animales estudiados
6	0,006 „ „ „ „	El 0 % de los animales estudiados
7	Insaponificable de germen de trigo (6)	El 100 % de los animales estudiados
8	Ninguno (7)	El 0 % de los animales estudiados

Key: 1. Group; 2. Supplement; 3. Normal histological spermatazoa conserved (in grams); 4. mg of tocopherol acetate; 5. % of the animals studied; 6. emulsion to wheat germ; 7. no supplement.

The preceeding annotated results indicate:

The minimal dose of vitamin E needed for preservation of normal histology of sperm is equal to 30 gammas of alpha tocopherol acetate.

This dose is the same minimal dose required to maintain normal fecundity and testecular weight.

With the object of comparing our results with K E. Mason's, we were intent on clarifying the three very different grades obtained on the scale by Mason (1926). We encountered the following facts:

1. Some animals in the same histological selection presented aspects that permitted clarification of three very different grades of Mason's scale.

2. In the group of animals that received 30 micrograms of vitamin E daily, 30% showed some little symptoms of histological alteration of the testacles which permitted classification in grade one on Mason's scale. These animals fertilized the females normally.

3. In the group that received 15 gammas of the vitamin, all the animals showed greater histological lesions of the testacles than grade 1. In three of the animals there was an alteration of a grade of 5B.

4. In the group that received 600 micrograms of alpha tocopherol daily, 66% of the animals presented histological lesions of the testacles corresponding to grade 1 of Mason's scale.

5. All the animals that received 6 micrograms of vitamin E showed a grade 5B lesion, the same as those which received no supplement.

The aforementioned annotations indicate:

- That the 30 micrograms daily dose of alpha-tocopherol acetate is sufficient to preserve normal histological spermatozoa in 100% of the animals that received it.

- That this dose did not prevent some symptoms of slight degeneration of the testacles.
- That the appearance of grade 1 in the histological table of the testacles on Mason's scale did not prevent the animals from reproducing normally (according to Mason in 1926).

Comment:

In the present work we studied the results obtained thru the study of the genital aparatus of 6 groups of animals which for a period of 26 weeks received a daily dose of alpha-tocopherol acetate of between 600 and 6 gammas. The state of the genital aparatus was determined by the following three methods:

- By the capacity to impregnate normal female rats.
- By the weight of the testacles.
- By the histological state of the testacles.

It was determined that a daily dose of 30 gammas of alpha-tocopherol acetate was sufficient to prevent sterility in 100% of the animals used and to prevent atrophy of the testacles and maintain histologically normal spermatozoa in 100% of the animals studied. It was not sufficient to prevent 30% of the animals studied to show a histological lesion corresponding to grade 1 on Mason's scale.

The fact that we were able to consult an interesting study published by Mason in 1940 before the publication of this work, induced us to investigate why there was a difference between his work and ours. When our work was finished it was not possible to take advantage of some of the initiatives proposed by this author. Therefore we have contented ourselves with observing our results and looking at them for the causes of the differences encountered.

These differences could be explained for two fundamental reasons:

1. By the use of animals with different reserves of vitamin E or differing weights.

2. By the differences in the value placed on the form of the normal state of the genital aparatus.

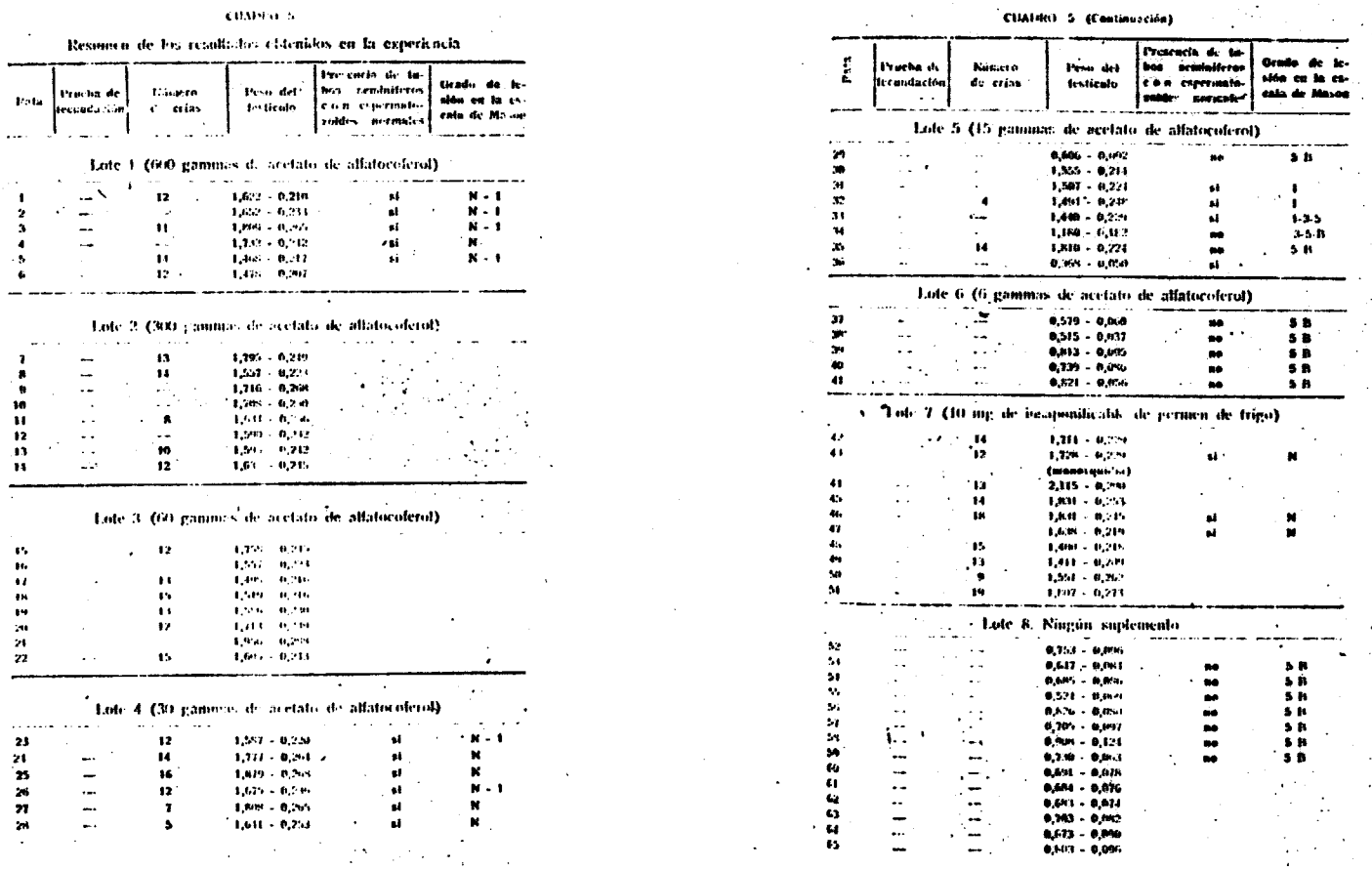
We previously said that our animals came from femals that received a diet rich in vitamin E, but that they never had access to the diet of their mothers. Mason & Byran in 1938 determined that the maximum reserves of Vitamin E that could accumulate in a rat during a period of lactation is the quantity sufficient to delay the appearance of the degeneration of the testacles by 20 to 30 days. In a later work Mason & Bryan (1940) permitted 47 days of protection to be conferred on the reserves of vitamin E acumulated during lactation. The reserves acumulated during gestation were unimportant according to the aforementioned authors. In agreement with what was said before, the maximum quantity of the vitamin that could accumulate in our animals during the period of lactation was sufficient to delay the appearance of the symptoms of testicular degeneration by 47 days. The lack of vitamin E produced in our animals was 47 days after that used by Mason's animals. This fact was compensated for during the major portion of our experiment because the aforementioned author began administration of the vitamin 10 days after the separation of the animals from their mother and interrupted them 20 days before the end of the experiment. The weight of the animals used by Mason was very much the same as those used by us.

Possibly the difference of dose used could be due to the methods used to determine what constituted normal genital aparatus. In our experiment we were guided by the principal of the animals ability to impregnate normal females while Mason solely used the histological table of the testacles as a criterion. In our experiment the animals that showed a grade of 1 on Mason's scale were fertile. Possibly the dose of vitamin E necessary to maintain normalcy on the histological table of the testacles is slightly more than that necessary to maintain the capacity to fecundate, avoid testecular atrophy, and maintain normal histological spermatazoa.

The animals that received 600 micrograms daily of the vitamin showed slight symptoms of testicular atrophy. This fact led us to believe that there existed a state of hypervitaminosis of vitamin E.

The fact that Mason used alpha-tocopherol and that we used alpha-tocopherol acetate does not negate what was done before. According to O. Isle (1938) alpha-tocopherol acetate is biologically as active as alpha-tocopherol and presents the advantage of being much more stable.

Table V Presence of seminal vesicles with normal spermatazoa.



Key: 1. Rat; 2. Test of fecundation; 3. Number in brood; 4. Weight of testacle; 5. Lesion grade on Mason Scale; 6. Group 1 (600 micrograms of tocopherol acetate; 7; Group 2 (600 micrograms of α-tocopherol acetate; 8. Group 3 (60 micrograms of α-tocopherol acetate; 9. Group 4 (30 micrograms of α-tocopherol acetate; 10. Group 5 (15 micrograms of α-tocopherol acetate; 11. Group 6 (6 micrograms of α-tocopherol acetate; 12. Group 7 (10 micrograms of wheat germ; 13. Group 8 No Supplement.

Conclusions:

1. The dose of 30 gammas of alpha-tocopherol acetate administered daily for six months is sufficient to maintain normal capacity for fecundation, avoiding atrophy of the testacles and maintaining normal histology of the testacles in 100% of the animals in the experiment.

2. This dose did not prevent the appearance of slight symptoms of testicular degeneration compatible with normal fertility.

3. It was suggested that the possibility of hipervitaminosis of vitamin E existed.

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The Effect of Vitamin E, Dehydrated Alfalfa Meal and Condensed Fish Solubles Upon Hatchability of Eggs from Broad Breasted Bronze Hens Maintained on Litter

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AN INCREASE in the hatchability of eggs from Empire White turkey hens due to the addition of vitamin E to the diet was reported by Jensen (1953). The effect of vitamin E upon the reproductive performance of Beltsville Small White hens, maintained on wire floors, was reported by Atkinson *et al.* (1955 a) and Ferguson *et al.* (1954). Dehydrated alfalfa meal, condensed fish solubles and other supplements were reported to be sources of unidentified factors by a number of workers (German *et al.*, 1948; Carlson *et al.*, 1949; Atkinson *et al.*, 1951, 1953, 1955 b; Heuser and Norris, 1951; Scott, 1951; Kohler and Graham, 1951; Stephenson and Clower, 1952; McGinnis *et al.*, 1952; Slinger *et al.*, 1952; Fuller *et al.*, 1952; Arscott and Combs, 1953; Halbrook *et al.*, 1953; Hansen *et al.*, 1953; Jacobs *et al.*, 1953; Fisher *et al.*, 1954; Patterson *et al.*, 1955; and others.)

The present experiment was designed to determine the effect of adding vitamin E, dehydrated alfalfa meal, and condensed fish solubles to the diet of Broad Breasted Bronze hens maintained on litter on the reproductive performance of such dams.

EXPERIMENTAL PROCEDURE

One hundred-twenty Broad Breasted Bronze turkeys hens were divided into eight groups of fifteen birds each. The birds had been reared to maturity on the range of the Texas A. and M. College Poultry Farm. The birds had been fed a practical diet containing adequate amounts of known nutrients, antibiotics and sources of unidentified growth factors. The hens were placed in pens (14′ × 14′) on litter in November, 1954, and lighted. The basal diet used in the present experiment (listed below), was fed to all groups for five weeks prior to the initiation of the experiment on January 19, 1955. The composition of the basal diet was as follows: 25% soybean oil meal, 35% ground yellow corn, 34% ground milo, 3.0% dicalcium phosphate, 2.5% oyster shell flour, and 0.5% salt. The following were added per pound of feed: 9,000 I.U. vitamin A (stabilized), 1,200 I.C.U. vitamin D_3, 6 mcg. vitamin B_{12}, 400 mg. choline chloride, 2 mg. menadione, 2 mg. riboflavin, 12.5 mg. calcium pantothenate, 20 mg. niacin and 160 mg. $MnSO_4$. The supplements to the basal diet for the eight groups are indicated in Table 1.

Eggs were collected daily, stored in a cooler at 55°F., and set at weekly intervals. The incubation procedure and analyses of the eggs for tocopherol content was the same as that reported by Atkinson *et al.* (1955 a).

RESULTS

Supplementation of vitamin E, dehydrated alfalfa meal and condensed fish solubles to the basal diet resulted in hatchability percentages varying from 71 to 79% during the first eight weeks of the test (Table 1). In each instance where vitamin E was added (groups 2, 4, 6 and 8) hatchability was increased 2 to 8% over that

TABLE 1.—*Hatchability of fertile eggs and tocopherol content of eggs from Broad Breasted Bronze turkey hens*

Group	Supplements to basal diet[1]	Hatchability (%)		Tocopherol (Mcg./yolk)[2]		Embryonic mortality[3]
		1–8 wks.	9–20 wks.	13 wks.	21 wks.	
1	None	74	56	430	540	24.3
2	Vit. E	77	60	1,820	920	30.4
3	DAM	71	60	520	370	26.3
4	DAM+Vit. E	79	68	630	1,250	21.6
5	CFS	76	68	520	840	19.9
6	CFS+Vit. E	78	68	1,170	1,100	18.8
7	CFS+DAM	74	68	690	580	18.5
8	CFS+DAM+Vit. E	76	77	1,180	1,050	8.0

[1] Vitamin E = DL-α-tocopheryl acetate, 20 mg./lb.; DAM = dehydrated alfalfa meal, 5%; CFS = condensed fish solubles, 5%.
[2] Pre-experimental tocopherol levels: 471 mcg./yolk.
[3] Fourth week embryonic mortality in eggs laid during weeks ninth to twentieth.

observed in groups 1, 3, 5 and 7. The latter groups represent the control group, dehydrated alfalfa meal, condensed fish solubles or combinations of these supplements, without added vitamin E.

The hatchability of eggs during the last twelve weeks of the experiment was improved from 4 to 12% by the addition of either vitamin E, dehydrated alfalfa meal or condensed fish solubles, fed singly or combinations of any two of these supplements. However, when all three of the supplements were fed (group 8), 77% hatchability was maintained which represented an increase of 21% over that of the basal group (group 1). Furthermore, the hens in this group were the only ones which did not show a decrease in hatchability during the ninth through the twentieth week. Analysis of the data for the 9-20 week period showed that the addition of vitamin E to the diet was statistically significant at the 5% level of probability. The feeding of condensed fish solubles produced an increase in hatchability which was significant at the 1% level of probability.

Embryonic mortality was essentially the same for eggs from all groups during the first eight weeks. Similarly, there was very little difference in the embryonic mortality between groups 1 through 8 during the first three weeks of the incubation period, of eggs laid during the 9th through the 20th week. Only 8% mortality was found in the fourth week of incubation in eggs from the hens in group 8 which were fed supplements of alfalfa meal, fish solubles and vitamin E (Table 1), while the embryonic mortality ranged from 18.5 to 30.4% in the remaining groups.

The tocopherol content of the egg yolks was increased when vitamin E was added to the diet of the dam (Table 1). The addition of condensed fish solubles or dehydrated alfalfa meal produced, essentially, no effect on the tocopherol content of the yolks. No explanation is readily apparent for the low value obtained in group 4 (13 weeks) or the slightly increased tocopherol in group 5 (21 weeks).

There was essentially no difference in egg production or fertility due to any of the supplements throughout the twenty-week period.

DISCUSSION

The above results are interesting in view of previous reports regarding the addition of vitamin E and unidentified factor sources to the diet of the turkey hen (Jensen, 1953; Atkinson *et al.*, 1955a; Ferguson *et al.*, 1954). Jensen reported an increase in hatchability of eggs when supplemental

vitamin E was added to the diet of Empire White turkey hens. The reports by Atkinson *et al.* and Ferguson *et al.* were to the effect that the addition of vitamin E resulted in an increase in hatchability of eggs from Beltsville Small White hens. The BSW hens were maintained on wire floors and were offspring of hens previously maintained on wire floors. In reversing the vitamin E treatments (Atkinson *et al.*, 1955 a), the tocopherol content of the eggs was likewise reversed, and while hatchability decreased to some extent, it did not drop as low as the tocopherol content indicated. It was suggested at that time that possibly an unidentified factor or factors were involved in the hatchability as well as vitamin E.

In the present study, the hens were maintained on litter and fed sources of unidentified factors, as well as vitamin E. The increase in the tocopherol content of the egg was due primarily to the vitamin E supplements. It is interesting also that in groups 2, 4, 6 and 8, which contained supplemental vitamin E, the hatchability of eggs during the first eight weeks was from 2 to 8% greater than was obtained in groups 1, 3, 5 and 7. In other words, when comparing group 1 with group 2, group 3 with group 4, group 5 with group 6 and group 7 with group 8, there is a definite increase in the hatchability of fertile eggs during the first eight weeks due to the addition of E to the diet, if the odd-numbered groups are considered as basal groups for the consecutive even-numbered groups.

The addition of dehydrated alfalfa meal to the diet produced only a slight increase in the tocopherol content of the egg. This is in agreement with the report by Singsen *et al.* (1955) to the effect that alfalfa was only 25% as effective in preventing encephalomalacia as an equal unitage of vitamin E from *d* tocopheryl acetate.

It would appear from these data that the addition of vitamin E to a practical type turkey breeder diet, fed to Broad Breasted Bronze turkey hens maintained on litter, results in an increased hatchability of eggs from the hens. However, greatest hatchability resulted when dehydrated alfalfa meal, condensed fish solubles and vitamin E were all present in the diet. The latter combination was able to maintain the level of hatchability, during the last 12 weeks of the 20 week test, equal to that obtained during the first eight weeks.

SUMMARY

Broad Breasted Bronze turkey hens maintained on litter were fed an all vegetable protein diet supplemented with various combinations of dehydrated alfalfa meal, condensed fish solubles and vitamin E for a period of twenty weeks.

A slight increase in hatchability was obtained during the first eight weeks of the experiment when vitamin E was added to the diet.

Dehydrated alfalfa meal, condensed fish solubles and vitamin E fed in combination in the diet, maintained hatchability at the same level throughout the entire twenty week study; this was 21% higher than the basal group during the ninth through the twentieth week. Supplements of any of the above when fed singly or combinations of any two resulted in a hatchability increase of from 4 to 12% over the basal group.

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THE ACTION OF VITAMIN E ON THE PROCESS OF BLOOD COAGULATION

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A factor of great biological interest, vitamin E, a little more than thirty years since its discovery (Evans, 1923), has prompted a large quantity of investigations and studies, and has been the theme of the discussions of at least three international congresses: London, 1939; New York, 1949; and Venice, 1955.

The physiological role of this vitamin takes place in nearly all aspects of the organic and mineral metabolism[1-9], influences the oxidative processes[10, 11], and internal secretions[12,31] reveals direct intervention in the regenerative processes[14] and is of fundamental importance for the normal trophism of some tissues: the connective, muscular and nervous in particular[15-17].

From the point of view of clinical applications, alpha-tocopherol, after its first uses in the field of sterility (Evans et al.) was found to have greatest indications in the treatment of peripheral vascular ailments, both inflammatory and degenerative. After the first observations of Dam[18] (1940), confirmed by Mason[19] and Shute[20], who demonstrated experimentally how a deficiency of vitamin E provokes an exudative diathesis with marked capillary fragility and a tendency toward edema, most further experiments agree in documenting several effects which the vitamin might have on the vascular structures, preserving the endothelia, moderating capillary permeability, stimulating the vascular neoformation in the tissues of cicatrization and organization, and finally, favoring the trophicity of the entire vasal wall.

Shute, Vogelsang, Skelton, Shulte[21], Enria and Ferrero[22], Butturini[23], Rateliffe[24], and many others report more or less numerous clinical statistics of good results obtained with the use of vitamin E in the most various vascular ailments, from phlebothrombosis to protopathic arteriopathies.

Recently, Calvi, Zampetti[25], and Raverdino[26], in separate observations, reported notable benefits in the treatment of retinal arterial spasms; Caleone and Minelli[27] in coronary claudication, and Rateliffe[24] in intermittent claudication. These spasmolitic actions can be explained by an improved irroration and a reordered trophism of the vasal walls. Recently, in our clinic as well, vitamin E in combination with other enzymes was administered endoarterially in cases of peripheral vasculopathies, and yielded favorable results (Feruglio and Nardini[28]).

Because of its protective effect on the endothelia and and its regulative influence on capillary permeability, the the vitamin has been used widely in cases of vasculopathic hemorrhagic syndromes.

Masure[29], Prosperi and Lottini[30] and others have reported good results in cases of recurrent epistaxes, hereditary hemorraghic telangiectasis, and in the vascular syndrome of Willebrand.

De Nicola[31] for his part has observed in animals with aviteminosis E. a hemorraghic state that is insensitive to vitamins C, K and P, and attributes this state to prevalently vasal lesions. Still in the experimental field, worthy of particular attention are the studies of Skelton, Shute, Skinnon and Waud[32], (1946), who cured thrombocytopenic purpura in the dog with the administration of alpha-tocopherol. Around the same time, Vogelsang obtained surprising results in the human. Treating a case of thrombocytopenic purpura with a daily dose of 200 mg vitamin E, the author not only observed the rapid cure of this hemorraghic state, but also a quick return of the platelets to normal values[33].

These last bibliographic indications already reveal how the interpretative orientation of the mechanism of action of vitamin E in the treatment of some purpuras can move the point of attack of the vitamin itself, from the wall of the vessel to the elements of the blood, and encourages thus an investigation of the possible and direct influences of the tocopherols on the plasmatic factors of coagulation.

Towards this end, the Italians Tropeano[34] (1948) and Prosperi and Lottini[30](1949), among the first, were able to document a certain action of alpha-tocopherol on the process of blood coagulation; the former succeeding in hastening the time of clotting, in a case of fibrinopenia treated with vitamin E, from 10-20 hours to 1 hours 20 minutes - 2 hours 20 minutes; and the latter obtaining a clear reduction of the time of clotting and the time of prothrombin activity in adult and child subjects submitted to treatment with alpha-tocopherol.

Recent analogous results have been indicated by many authors (Salvini and Scardigli[35], 1952; Prosperi and Bonselli[36], 1953), and there have been many attempts as well at establishing which annulus of the complex chain of blood clotting it is, that the effect of alpha-tocopherol might interfere.

The literature of the past five years offers a confusion of scientific results and hypotheses, often in contradiction with one another.

Along with the authors who have striven to justify an accelerating effect of vitamin E on the process of blood clotting, by saying it causes an increase in thrombokinase or prothrombin[30] activity or in the number of platelets[20, 32], there are those who would demonstrate an absolute anti-coagulative activity of vitamin E[38,39], detailed above all in an increase of antithrombin[40]. Because of this supposed action of vitamin E, especially in the North American countries, it has been widely used in the prophylaxis of post-operative phlebothromboses[38,41].

Marcacci and Camera[42], (1954), and De Nicola[31](1955), on the basis of their observations, maintain that the apparently contradictory behavior of vitamin E is bound to the type of solification

of the vitamin itself, in the sense that in the form of an acetate it would have an accelerating effect, and in the form of a phosphate it would have a depressing effect on the coagulation of blood.

Finally, there are also authors (Paul, J.A. Lewis, De Luca[43]) who deny any effect whatever of alpha-tocopherol on the coagulation of the blood.

The present study, making use of the actual known facts on the mechanism of clotting and of new methods of study, by examining the single stages of the process of blood clotting, is intended to be a contribution to this vastly debated question of the real effects of alpha-tocopherol on the coagulation of blood.

CONDITIONS AND METHODS

Towards the above-named end, we examined three groups of patients, all of the male sex and of ages between 18 and 56 years, but free from any diseases which might have an influence on blood clotting and subjected to to indifferent therapies; to these patients we administered alpha-tocopherol in the following doses and manner.

To a first group of five patients, the pharmaceutical was administered orally at a dose of 100 mg per day for seven days.

To a second group of patients, alpha-tocopherol was administered intramuscularly at a dose of 50 mg per day for 10 days.

Finally, for the purpose of studying the immediate modifications induced by the pharmaceutical, we chose the intravenous route in a third group of 4 patients, to whom a single dose of 100 mg was administered.

The blood clotting analyses which we performed were the following:

1) time of coagulation of the blood in toto (Lee-White method);
2) plasmatic prothrombin activity (Quick method);
3) recalcification or Howell time;
4) serous prothrombin activity (residual prothrombin);
5) antithrombin activity;
6) blood platelet count.

In the first two groups of patients, the analyses were made of blood samples withdrawn at the beginning and end of the treatment. In the third group these analyses were made of four samples of blood withdrawn, respectively, before and after 10, 30, 60, 90 minutes from the intravenous injection of the alpha-tocopherol.

The results obtained are summarized in the table.

RESULTS AND CONCLUSIONS

From the data reported in the table it can be seen, in the first place, how during treatment with vitamin E the total time of coagulation of the blood is, in nearly all the cases, constantly shortened, regardless of the route of administration (oral, intramuscular, or intravenous).

This contraction of the time of coagulation goes from a minimum of 9% to a maximum of 30%.

Only in two cases did the time of coagulation appear unmodified or even extended (cases 5 and 10) with variations less than 10%.

The explanation of this behavior of the clotting time is supplied by the analysis of the other data reported in the table; in particular of the Howell time or recalcification time (the expression of the thrombopastin activity of the plasma) (De Nicola) and the antithrombin level, the only blood clotting tests which, in our research, yielded significant changes from the initial values. (The prothrombin activity on the plasma and the serum after 4 hours, and the platelet count, showed no significant deviations after the administration of the alpha-tocopherol).

The Howell time appeared to be slightly shortened in all the cases we studied. The reduction values varied between a maximum of 21% and a minimum of 6%. They are caused by an increased thromboplastin activity of the plasma, which occurs after treatment with tocopherol.

This is in accord with the contraction of the clotting time which has already been indicated, and both tend to confirm the experimental data already reported by Troncano et al.[41], Prosperi and Borselli[36], Scevola[45].

The antithrombin rate often appeared to be increased (only in two cases is it slightly diminished) from minimum values of 4% to maximum values of 71%.

This contrasts with the preceding data in that it is the expression of a hypocoagulative attitude. The investigations of Masure and Ruyssevelt[46] also report an increase in the antithrombin factor.

Obviously the contrast between the hypocoagulative effect which derives from such an increase of antithrombin and the hypercoagulative one bound to the increase of thromboplastin activity can be overcome if we consider that this latter action can be able to neutralize the former and, if it prevails, to have the ultimate effect of reducing the clotting time.

Sometimes, it may be that the antithrombin activity dominates the entire coagulating process, yielding as a result an extended clotting time, as in the two cases which we have described.

This twofold action of vitamin E on the blood clotting process and the resulting capacity to yield effects in clear contradiction, was without doubt the reason that even to this day the various authors have been separated into two opposing camps: the one attributing a hypocoagulative capacity to tocopherol, the other affirming the contrary thesis.

In our research, we definitely found a prevalence of the hypercoagulative effect of the vitamin, especially in the subjects treated intravenously or orally. Nonetheless, we are far from assuming a particular position and, like Prosperi and Borselli[36] and other authors[47,48], from admitting a relative danger in the use of the vitamin in cases of thrombotic vasculopathies.

The brilliant results reported by a large group of observers, concerning the beneficial intervention of the tocopherols in thrombophlebitis, thromboarteritis and phlebothrombosis, can always be justified by the documented protective and eutrophic action of

the vitamin on the endothelia and on the structure of the interior of the vasal wall.

Because of this very important effect the vitamin, despite its prominent hypercoagulative activity, is still used with favorable results, along with other enzymes and vascular medicines, in our clinic as well, in the treatment of peripheral angipathies.

SUMMARY -- The study of the action of vitamin E on blood clotting has demonstrated, in nearly all the cases and regardless of the route of administration (oral, intramuscular or intravenous), on the one hand a shortening of the clotting time and an increase of the thromboplastin activity, determined by the Howell time and the residual prothrombin, and on the other a clear increase of plasmatic antithrombin. (No modification appeared in the plasmatic prothrombin activity and the platelets.) The apparent contradiction of the results obtained may offer an explanation of the discordant conclusions reached by the numerous authors who have dedicated themselves to the argument. In conclusion of our investigation we retain that vitamin E details in the complex a hypercoagulative effect, the increase of plasmatic antithrombin being generally insufficient to neutralize the other action of the tocopherols, the one directed to exaltation of the thromboplastin activity of the plasma.

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STUDIES ON CHANGES IN FLOUR TOCOPHEROLS FOLLOWING AGEING AND TREATMENT OF THE FLOUR WITH CHLORINE DIOXIDE

By A. C. FRAZER and J. G. LINES

The tocopherols in freshly milled flour were measured using two-dimensional chromatography. Total tocopherols were in the range 1500–1600 μg/100 ml, but only 17–18% of this was α-tocopherol. Aerobic storage for 190 days caused a reduction of all tocopherols by 62–67%. Treatment of the flour with chlorine dioxide at the usual commercial level caused a comparable reduction, averaging 58%; ε-tocopherol was rather less affected than α-tocopherol.

Studies in human volunteers indicated that a daily supplement greater than 10 mg of tocopherol was needed to bring about a significant change in the level of α-tocopherol in the blood. When bread made from untreated and chlorine dioxide-treated flour was fed to groups of volunteers in a double-blind cross-over trial for 50 days on each type of bread, no significant correlation could be found between blood tocopherol levels and the type of bread consumed. To examine whether a longer period of time on the diet was necessary, the blood tocopherol levels of thirteen English students who had habitually consumed bread made from treated flour were compared with those of thirteen French students, matched for age, who had for several years consumed French bread which is made from untreated flour. The analyses were carried out in Birmingham and in Paris by the same investigator. No significant differences in tocopherol levels were found. It is concluded that the consumption of bread made from flour treated with chlorine dioxide will have no significant effect upon the blood level of α-tocopherols under the dietary conditions at present prevailing in Britain.

Introduction

Flour improves its baking qualities on storage; this is largely due to oxidative changes. This ageing process can be artificially by applying oxidising agents to the flour. Flours vary considerably in composition according to variety and to the climatic conditions under which the wheat was grown. The different types of flour require different quantities of oxidising agents which can be conveniently applied as a gas to the flour stream. The use of a gaseous oxidising agent has several advantages over simple storage of flour; it is more easily measured and controlled, it saves time and storage space and it is easier to adjust the extent of oxidation to suit the characteristics of the flour. Extensive studies have been carried out on flour treated with chlorine dioxide and it has been shown that the biological properties of chlorine dioxide-treated flour and bread made from it are not significantly different from those of similar materials untreated, except with regard to tocopherol content. Chlorine dioxide treat-

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ment of flour caused significant destruction of tocopherols and these may well be less in quantity than the tocopherol content of bread made from untreated flour.[1,2]

The object of the studies reported in this paper was to obtain more detailed information about the changes in tocopherols that occur on ageing and on treatment with chlorine dioxide and to attempt to evaluate the possible significance of such changes in bread when it is consumed by human subjects.

Materials and methods

Untreated Flour

The flour used in these studies was supplied to us by Messrs. Wallace & Tiernan Limited. The untreated flour was composed of 50% Top Patent, 30% Middle Grade, 16% Low Grade and 4% Bottom Grade. Creta and Vitamin Mastermix were added.

Treated Flour

The same flour was treated with chlorine dioxide at an average level of 1·69 g per sack (280 lbs). The individual rates per sack for the divisions of the flour stream were: 50% Top Patent 0·5 g, 30% Middle Grade 2 g, 16% Low Grade 3·5 g, and 4% Bottom Grade 7 g. The level of treatment was independently checked in the laboratories of Messrs. Kent-Jones and Amos.

Bread

The bread was baked from untreated and treated flour under identical conditions and supplied direct to the volunteers by post. The arrangements for preparation and supply of bread were made by Dr. D. W. Kent-Jones.

Reagents

Standard reagents as defined in the appropriate reference cited were used throughout.

Volunteers

The volunteers were healthy male graduate or undergraduate students or technicians.

Blood tocopherol estimation

Alpha tocopherol constituted more than 75% of the total tocopherols in plasma;[3] it occurred almost exclusively in the unesterified form[4] and it was the most biopotent tocopherol.[5] Blood α-tocopherol was conveniently expressed as free α tocopherol concentration. The method for estimation of plasma α-tocopherol consisted of one-dimensional paper chromatography of a plasma lipid extract followed by elution of the α-tocopherol band and colorimetric determination of the α-tocopherol by the Emmerie-Engel reaction.[6,7] The results reported were in all cases the mean results obtained from duplicate or triplicate analyses.

Estimation of flour tocopherols

Flour tocopherol analyses were carried out using the method published by the Vitamin-E Panel of the Analytical Methods Committee of the Society of Analytical Chemistry.[8] In this method two-dimensional paper chromatography was used to separate the four tocopherols (α, β, ϵ, ζ) occurring in flour. Each spot was eluted and quantitatively determined by the Emmerie-Engel reaction. The analysis of both flour tocopherols and plasma α-tocopherol were carried out in the dark in an atmosphere of nitrogen, to prevent oxidation of the tocopherol.

Results

Effect of ageing on flour tocopherols

Fresh untreated flour was stored in an open tin, so that 1 kg flour a surface of about 150 sq. cm was exposed to atmosphere. At intervals up to 190 days, samples flour were analysed for their tocopherol content. Of the tocopherols present in flour, β and ζ tocopherols occur the smallest quantities and as they are also close together the chromatograph paper, they were estimated together. The proportion of β to ζ is usually about 3 : 1. Replicate analyses were made on each flour sample studied. The results are shown in Fig. 1 and Table I.

The natural aerobic oxidation under the conditions described caused the destruction of about 63% of the total tocopherols in 190 days. Each form of tocopherol was destroyed to about the same extent.

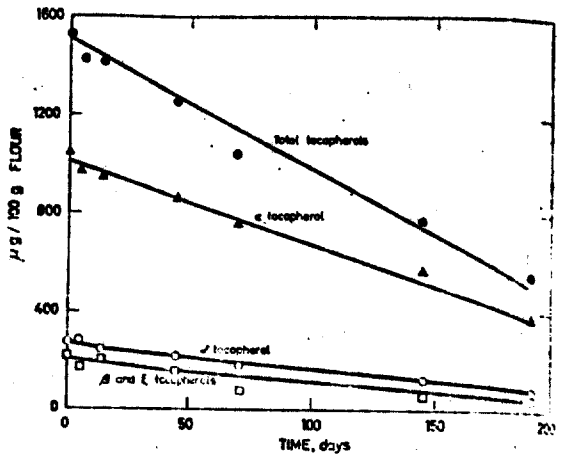

FIG. 1. *Illustrating the loss of tocopherols from flour on storage in the presence of air*

TABLE I

Effect of ageing on tocopherol content of flour

	Average amount of tocopherol (μg/100 g flour) on day: 1	190	% destroyed in 190 days
α	273·2	95·5	65·1
ε	1037·4	385·6	62·7
β & ζ	217·8	73·1	64·1
Total	1528·4	560·1	63·4

The effect of chlorine dioxide treatment on the tocopherol content of flour

Samples of untreated flour and flour treated with chlorine dioxide were studied in the same way. The results are given in Table II.

The treatment of flour with chlorine dioxide caused destruction of all tocopherols. The extent of the reduction in tocopherols was of the same order as that observed following storage in the presence of air for six months.

TABLE II

Effect of chlorine dioxide treatment of flour on tocopherol content

	Tocopherols μg/100 g flour			
	α	ε	β & ξ	Total
...rated (Six samples)				
...verage	257	998	256	1512
...nge	221–300	907–1064	135–300	1400–1550
...total	17·2	66·2	16·6	100
...ne dioxide treated ... samples)				
...verage	43·3	497·3	83·9	629·5
...nge	40–80	473–537	82–101	570–699
...total	6·9	79·0	14·1	100

...s in man of tocopherol ingestion

...fects of supplementation with α-tocopherol on the blood ...opherol levels in man

...) The first experiment on human subjects was to deter... the fasting plasma α-tocopherol in three normal volun... at intervals over a period of two months. After the base... had been established, a daily supplement of 50 mg ...opherol dispersed in butter was given to each of the ...ts and the fasting plasma α-tocopherol concentration ...termined. After several days, the α-tocopherol supple...ation was stopped in one of the subjects and was reduced ...e other two, first to 25 mg and then to 10 mg daily. The ...ma α-tocopherol levels were also determined in all three ...jects after the tocopherol supplement was removed. The ...t on blood α-tocopherol in two of these subjects is ...aked in Fig. 2. The third subject gave a similar picture. ...can be seen from these results that there was considerable ...y variation in the fasting plasma α-tocopherol concentra... in each individual subject. This may have been due in ...to variations in the daily intake of tocopherols. These ...lts also demonstrated that a daily α-tocopherol supple...t of 50 mg caused a significant rise in the blood α-toco...rol level; the elevated concentration was maintained when ...supplement was reduced to 10 mg daily.

...) The next step was to determine how much α-tocopherol ...s needed as a daily supplement to raise the blood α-toco...rol level significantly. The normal ranges of fasting plasma α-tocopherol levels were determined in a number of normal volunteers on a low tocopherol diet. Then, tocopherol supplements were given to each subject in the form of α-tocopherol acetate in gelatine capsules once daily following a main meal. After an equilibration period of 7 days, the plasma α-tocopherol levels were re-determined in the ensuing 5 weeks, during which time the daily supplement was continued. The results of these tests are given in Tables III and IV.

Supplementing the diet with 25 mg α-tocopherol acetate daily resulted in a significant rise in the blood α-tocopherol level. With one exception, a significant rise was not observed if the supplement was only 10 mg daily or less. Although the

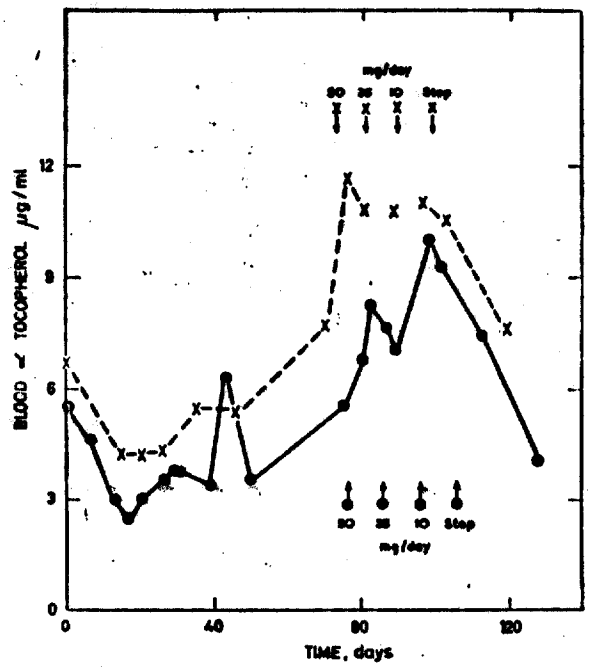

FIG. 2. *Blood α-tocopherol levels in two human volunteers showing effect of dietary supplementation with α-tocopherol*
(Note: Increase with 50mg/day maintained by 10mg/day. Rapid decline when supplementation stopped).

TABLE III

Plasma α-tocopherol levels in thirteen volunteers whose diets were supplemented with different daily intakes of α-tocopherol
(The figures shown are for each volunteer. The figures in parentheses show the number of blood samples studied in each instance)

Subject	Control period			α-Tocopherol supplement			
	Average	Range	S.D.	mg/day	Average	Range	S.D.
D.R.	4·2 (9)	2·6– 4·7	1·1	0	5·8 (7)	4·7– 7·8	1·0
E.P.	4·4 (9)	2·9– 6·5	1·1	0	4·5 (8)	4·3– 7·8	1·4
J.W.	5·7 (9)	4·5– 7·4	1·1	0	5·2 (9)	3·7– 7·6	1·4
B.C.	5·0 (9)	2·1– 7·5	1·5	3	6·8 (8)	4·6– 8·2	1·3
B.L.	3·5 (8)	2·3– 5·7	1·2	3	6·7 (8)	5·2– 8·7	1·4
R.C.	5·4 (9)	4·1– 7·1	1·1	3	6·3 (7)	5·3– 8·0	1·0
G.B.	5·5 (8)	3·5– 7·8	1·5	5	5·9 (8)	4·8– 7·5	0·9
M.S.	6·0 (11)	4·2– 9·4	1·7	5	6·2 (7)	5·6– 6·6	0·3
N.B.	4·5 (9)	2·1– 6·9	1·6	10	4·7 (7)	3·2– 5·9	1·1
P.S.	7·0 (9)	4·1–10·3	2·4	10	8·4 (9)	7·1–10·0	1·0
J.M.	3·4 (12)	1·8– 4·8	1·0	25	7·1 (9)	5·6– 8·7	1·4
J.D.	4·8 (9)	2·9– 8·0	1·8	25	5·8 (9)	4·3– 7·5	1·1
K.J.	4·7 (8)	3·4– 6·3	1·3	25	6·4 (9)	4·9– 7·5	1·0

TABLE IV

Plasma α-tocopherol levels in subjects whose diets were supplemented with different daily intakes of α-tocopherol
(The figures shown are group averages. The figures in parentheses show the number of volunteers in each group)
(*Further detail as shown in Table III*)

Group	Control period μg/ml	Control period S.D.	Supplemental period μg/ml	Supplemental period S.D.
No supplement (3)	4·8	1·3	5·3	1·5
3 mg/day (3)	4·7	1·5	6·6	1·2
5 mg/day (2)	5·7	1·6	6·0	0·7
10 mg/day (2)	5·7	2·3	6·8	2·2
25 mg/day (3)	4·2	1·5	6·4	1·2

increase in α-tocopherol levels in these studies was not statistically significant, the levels in the supplemental groups were almost invariably higher than those in the control groups. When all the blood α-tocopherol estimations made over a long period were examined, a consistent trend towards higher levels in the Spring as compared with the Autumn was observed. The control and supplemental groups were studied at a different time of the year—the control group in the Autumn and the supplemental group in early Spring. Thus, the rather higher levels observed in the supplemental group may have been due to a seasonal effect.

Since the total contribution to the dietary α-tocopherol of fresh flour was of the order of 1 mg a day or less, it did not seem likely that any alteration in this contribution would significantly affect blood α-tocopherol levels. The studies so far had emphasised the wide fluctuations in blood α-tocopherol levels observed in healthy individuals. To complete these studies, it was decided that a comparison should be made of the blood α-tocopherol levels in individuals consuming breads made from untreated and treated flours.

Effects of different breads made from treated and untreated flour

(*a*) The first experiment on the effects of different bread was conducted on 11 healthy males aged between 18 and 26 years. Throughout the duration of the experiment, a detailed record of their daily food intake was kept. The intake of all foodstuffs rich in tocopherols was discouraged. To familiarise the volunteers with this regimen, a month was allowed to elapse before supplying the experimental bread.

The 11 volunteers were divided into two groups, one group receiving bread baked from untreated flour and the other similar bread made from flour treated with chlorine dioxide. After 50 days, the supply of treated and untreated breads to the two groups was reversed. Fasting plasma α-tocopherol concentrations were determined at approximately 10-day intervals throughout the experimental period. The results are shown in Table V.

It will be seen from Table V that no significant differences were observed in blood α-tocopherol levels in this double blind test in volunteers consuming bread made from untreated or from treated flour over periods of 50 days.

(*b*) The second experiment in this series was a comparison of the blood α-tocopherol levels in a group of 13 young English males, who had always eaten English bread treated with improving agents, with those of a group of 13 French males, matched with regard to age, who had always eaten French bread in the preparation of which oxidising additives were not used. The English students were studied in

TABLE V

Blood α-tocopherol levels in two groups of subjects
(Volunteers C.1–C.6 consumed bread made from untreated flour for the first 50 days and thereafter the bread made from treated flour. Volunteers C.7–C.11 took the breads in the reverse order. Neither the volunteers nor the observers knew which bread was being consumed by each volunteer.)

Subject	Untreated flour Average μg/ml	Untreated flour Range μg/ml	Treated flour Average μg/ml	Treated flour Range μg/ml
C.1	5·6(4)	4·2–8·7	4·4(3)	3·3–5·3
C.2	6·2(3)	6·0–6·6	5·8(4)	4·6–6·8
C.3	6·8(4)	6·0–7·8	4·5(3)	2·1–6·9
C.4	5·4(4)	4·4–6·5	6·7(3)	3·4–9·1
C.5	3·7(3)	2·3–4·8	4·8(3)	4·5–4·6
C.6	5·7(4)	3·9–7·7	4·9(3)	4·4–5·6
	Treated flour		**Untreated flour**	
C.7	7·0(3)	6·4–7·7	6·7(3)	5·8–6·8
C.8	6·6(2)	6·5–6·7	4·7(3)	3·45–5·10
C.9	6·2(3)	5·4–7·3	5·5(3)	4·8–6·4
C.10	6·3(3)	5·9–6·5	4·9(4)	4·1–5·6
C.11	7·4(4)	5·2–8·3	7·2(4)	6·8–8·2

Birmingham and the French students in Paris. It was calculated that the French group received about 300 μg α-tocopherol and 1300 μg total tocopherols more each day from bread than the English group. The same methods and apparatus were used by the same analyst in both Birmingham and Paris. The results are shown in Table VI.

It will be seen from Table VI that, although the French group may have received about 300 μg more α-tocopherol daily from bread than the comparable English individual, no significant differences in the blood α-tocopherol levels between these two groups were found.

Discussion

These studies showed that there was a steady decline in the tocopherol content of flour on storage, more than 60% being destroyed in 190 days. The different tocopherols were reduced about equally. Treatment with chlorine dioxide caused destruction of a similar order, although the destructive effect on α-tocopherol was rather more marked than the effect on ε-tocopherol. It has been shown that baking resulted in a further reduction in the tocopherol levels.[9]

The proportion of the total dietary α-tocopherols that might be supplied by flour and bread was not more than about 900 μg (i.e., about 5–10% of the daily requirement) and usually it was less than this.

The studies in volunteers indicated the considerable variation that may occur in the blood α-tocopherol levels of an individual from day to day. In order to bring about a significant change in blood α-tocopherol levels, daily supplementation in the region of 10 mg daily was needed. It seems unlikely, therefore, that a small percentile reduction, or indeed, even complete destruction, of the tocopherols in flour or bread made from it, could be regarded as being nutritionally important, since the change in overall tocopherol intake would be no greater than the daily variations commonly

TABLE VI

Comparison of blood α-tocopherol levels in two groups of males, matched with regard to age
(The English group had consumed bread made from flour treated with oxidising maturing agents for many years; those in the French group had consumed French bread for many years in the preparation of which oxidising maturing agents had not been used. The analyses were carried out by the same observer using the same methods and apparatus. The numbers in parentheses show the number of samples analysed in each instance.)

ENGLISH GROUP				FRENCH GROUP			
	Average	Range	S.D.		Average	Range	S.D.
N.B.	4·5 (9)	2·1–6·9	1·6	J.B.	5·0 (8)	4·3–6·2	0·7
G.B.	5·5 (8)	3·5–7·8	1·5	S.B.	4·4 (7)	4·3–5·6	0·7
R.C.	5·4 (9)	4·1–7·1	1·1	B.D.	5·0 (12)	3·9–6·6	0·9
R.C.[1]	5·0 (9)	2·1–7·5	1·5	J.F.	2·6 (9)	1·2–3·9	1·0
J.D.	4·8 (8)	2·9–8·0	1·8	M.G.	3·5 (11)	2·2–4·5	0·7
K.J.	4·7 (7)	3·3–6·3	1·3	A.J.	2·3 (14)	1·0–3·4	0·8
B.L.	3·5 (7)	2·3–5·7	1·2	G.L.	2·8 (10)	1·2–3·9	0·9
J.M.	3·4 (11)	1·8–4·8	1·0	J.M.	5·8 (12)	4·3–8·3	1·2
K.P.	4·4 (9)	2·9–6·5	1·1	M.M.	4·8 (9)	3·6–6·1	1·0
D.R.	4·2 (9)	3·1–4·7	1·1	P.S.	4·5 (8)	2·9–6·3	1·2
M.S.	6·0 (11)	3·1–9·4	1·7	J.T.	5·6 (9)	4·7–6·9	0·7
R.S.	7·0 (9)	4·1–10·3	2·4	M.T.	7·2 (13)	5·6–9·3	1·2
J.W.	5·7 (9)	4·5–7·4	1·1	P.V.	3·3 (8)	2·6–3·7	0·4
Group totals	4·9 (125)	1·8–10·3	1·7	Group totals	4·4 (130)	1·0–9·3	1·7

countered. This was borne out by the further double-blind studies carried out in volunteers consuming for periods of 30 days bread made from treated or untreated breads. No significant effects on blood α-tocopherol levels were observed as a result of changing from one bread to another. To study possible longer term effects, the blood α-tocopherol levels were compared in a group of English students who had for some years consumed bread made from treated flours and a group of French students who had for some years consumed bread made from untreated flour. The students were matched with regard to age. No significant difference was found between the two groups.

The need for the use of an oxidising maturing agent for flour to be used in this country for bread-making has been discussed by Dodds.[10] The safety-in-use of chlorine dioxide for this purpose has been demonstrated.[1,2] The effect of treatment on tocopherol levels has been known for many years and this has been confirmed. It may be concluded from the studies in volunteers that the alteration in flour tocopherols that may be brought about by storage or the use of chlorine dioxide in the mill, has no significant effect on blood α-tocopherol levels. This would seem to indicate that the treatment of flour by chlorine dioxide has no important effect on overall α-tocopherol intake on a long-term basis and that the effect of chlorine dioxide treatment or ageing on flour tocopherols has little nutritional significance in man living under conditions similar to those existing today in this country.

The reduction of flour tocopherols to a low level will permit earlier development of oxidative rancidity in the flour lipids. This has been demonstrated in flours over-treated with chlorine dioxide.[2,11] The reduction of total tocopherols by treatment with chlorine dioxide demonstrated in these studies is of the same order as that observed with storage for 120–150 days. The greater reduction of α-tocopherol is not of significance in relation to development of rancidity, since α-tocopherol is just as powerful an antioxidant. The development of rancidity in flour or bread does not represent a serious hazard to the consumer, since it is associated with the development of off-flavour, which is likely effectively to prevent consumption of any great quantity of the deteriorated materials.

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THE EFFECT OF ANTIOXIDANTS ON POISONING BY CARBON TETRACHLORIDE

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SUMMARY.

The prior administration of the antioxidants α-tocopherol acetate, sodium selenite or NN¹-diphenyl-p-phenylenediamine (DPPD) afforded protection to rats given lethal doses of carbon tetrachloride.

Under the experimental conditions DPPD appeared to be the most effective of the three antioxidants tested. A single dose of 100 mg. DPPD injected intraperitoneally at the same time as CCl_4 was administered by stomach tube proved more effective than one dose of 95 international units of vitamin E given simultaneously with CCl_4, or than a dose of 95 I.U. given simultaneously and another five hours later. Sodium selenite was less effective than the other two antioxidants.

When antioxidants were given, pyridine nucleotide concentrations in the liver were maintained despite the subsequent administration of CCl_4, but in their absence concentrations of both oxidized and reduced pyridine nucleotides in the liver fell by more than 50 p.c. The loss of reduced pyridine nucleotides was completely prevented by DPPD, the only antioxidant studied in this regard, and the loss of oxidized pyridine nucleotides was largely prevented by DPPD or α-tocopherol acetate and to a lesser degree by sodium selenite.

Three doses of 100 mg. DPPD at 48, 24 and 0 hours before rats were given 4 ml. CCl_4/kg. prevented necrosis of liver cells and diminished the fatty change appreciably. A single dose of 100 mg. DPPD injected intraperitoneally 48 hours before 4 ml. CCl_4/kg. also largely prevented hepatic necrosis and decreased the fatty change.

INTRODUCTION.

The administration of either of the precursors of pyridine nucleotides, nicotinic acid or tryptophane, protects sheep and rats against the lethal and biochemical effects, and the death of liver cells which result from large doses of carbon tetrachloride (Gallagher and Simmonds, 1959; Gallagher, 1960a, b; Gallagher, Kondos and Southcott, unpublished data). The precursors stimulate pyridine nucleotide synthesis (Williams, Feigelson and Elvehjem, 1950; Chaloupka, Williams, Reynolds and Elvehjem, 1957; Preiss and Handler, 1958a, b), and normal levels of cellular and mitochondrial pyridine nucleotides are thus maintained despite administration of CCl_4 (Gallagher, 1960a, b). In the absence of prior treatment with the precursors CCl_4 causes a profound fall in the concentration of both mitochondrial and total cellular pyridine nucleotides.

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It had already been shown (Gallagher, 1960c) that a primary damaging effect of CCl_4 on mitochondria does not directly cause the passive loss of pyridine nucleotides but initiates a chain of events which, during active mitochondrial respiration *in vitro*, leads to the loss of mitochondrial pyridine nucleotides. Therefore it was considered that CCl_4 poisoning *in vivo* might be prevented, or successfully treated, by the use of agents blocking this later chain of events. The chelating agent ethylenediaminetetraacetic acid (EDTA) prevents the loss of pyridine nucleotides and maintains the normal oxidative capacity of washed mitochondria damaged by prior incubation *in vitro* with CCl_4 (Gallagher, 1960c). However, the administration of disodium EDTA does not protect rats from the lethal effects of large doses of CCl_4 (Gallagher, 1960a) but it seemed possible that the use of other substances capable of preventing the loss of mitochondrial pyridine nucleotides might protect.

Rees, Spector and Sinha (1961) observed that the antihistamine, Phenergan (10-(2-dimethylaminoisopropyl)-phenothiazine hydrochloride), when administered with and after CCl_4 protects the liver cells against necrosis. Subsequently it was shown (Gallagher and Rees, 1960) that Phenergan also prevents the fall in liver cell and mitochondrial pyridine nucleotides associated with CCl_4 poisoning. It seems likely that Phenergan prevents the loss of pyridine nucleotides by inhibiting some secondary reaction following CCl_4 damage to lipo-protein membranes. Phenergan has also been shown by McLean (1960a, b) to serve the same purpose as vitamin E in the prevention of dietary liver necrosis in rats and in the restoration of the ability of damaged liver slices to re-accumulate potassium. Earlier, Gallagher, Gupta, Judah and Rees (1956) had found that Phenergan prevents necrosis of liver cells and the accumulation of calcium within the liver cells in rats poisoned with thioacetamide. This protective effect against diverse causes of liver damage suggested that Phenergan acts on a general metabolic mechanism concerned with the maintenance of cellular and mitochondrial integrity.

Its value as a substitute for vitamin E indicated that the action of Phenergan might be associated with its antioxidant properties and that antioxidants might prove to be effective prophylactic or therapeutic agents in CCl_4 poisoning. In the current investigation, therefore, the antioxidants (Zalkin, Tappel and Jordan, 1960; Schwarz, 1958) α-tocopherol acetate, sodium selenite and NN¹-diphenyl-p-phenylenediamine, have been evaluated as protective agents in CCl_4 poisoning.

Materials and Methods.

Animals. Female Wistar rats weighing 150-180 gm. and fed a normal stock diet were used throughout the experiments.

Reagents. Carbon tetrachloride of reagent grade was further purified by distillation. α-tocopherol acetate (1 mg. = 1 international unit), sodium selenite, NN¹-diphenyl-p-phenylenediamine and alcohol dehydrogenase were of the highest quality obtainable commercially.

Methods.

The extinction coefficient at 260 mμ (E_{260}) of acid extracts of liver homogenates was measured in the following manner. Rats were killed by decapitation after stunning. The liver was removed rapidly and immersed in ice-cold 0·25 M sucrose solution. A 5 p.c. (w/v) homogenate of the liver in ice-cold 0·25 M sucrose was prepared immediately in a glass homogeniser. Duplicate 0·5 ml. samples of this homogenate were transferred quickly to glass centrifuge tubes containing 4·5 ml. of 5·56 p.c. trichloroacetic acid (TCA) at 0° C. to give a final volume of 5 ml. of 5 p.c. TCA. The tube contents were mixed by means of glass rods, allowed to stand at room temperature (20-24° C.) for 10 minutes, and were then centrifuged at 2000 r.p.m. in a M.S.E. Multiplex centrifuge for five minutes. The supernatant was collected and the E_{260} value was measured with a Beckman model D.U. spectrophotometer using a blank of 5 p.c. TCA.

The extinction coefficient at 340 mμ (E_{340}) of alkaline extracts of liver homogenates was measured in the following manner. Rats were killed by stunning and decapitation. Portions of liver (about 2 gm.) were removed, weighed rapidly and immersed in ice-cold 0·25 M sucrose solution containing 0·1M nicotinamide. Each liver sample was then washed, minced with scissors and homogenized in a Potter glass homogenizer to give a 2 p.c. homogenate in 0·25 M sucrose solution containing 0·1M nicotinamide. One ml. of ice-cold 1 N NaOH was added, whilst mixing with a glass rod, to 9 ml. of the liver homogenate to give 10 ml. of 0·1 N NaOH in the sucrose/nicotinamide homogenate.

The homogenates were extracted in this manner for 30 minutes, then centrifuged at 34,500 × g for 20 minutes. The supernatants were collected and the E_{340} value of each was determined against a blank of 9 ml. 0·25 M sucrose/0·1M nicotinamide and 1·0 ml. 1 N NaOH using a Beckman model D.U. spectrophotometer.

Reduced diphosphopyridine nucleotide (DPNH) was measured by the method of Spirtes and Eichel (1954) using alcohol dehydrogenase.

The dry weight of each liver homogenate was determined by extracting a 30 ml. sample three times with a mixture of boiling ethanol and diethyl ether (3 to 1, v/v) to remove lipids, drying in an oven at 100° C. for 24 hours, and then cooling in a desiccator before weighing.

Undiluted CCl_4 was introduced into the stomach with a syringe attached to a metal stomach tube while rats were anaesthetised lightly with diethyl ether. The dose was 4·0 ml./kg. or 2·7 ml./kg. as indicated. Earlier studies (Gallagher, 1960a) had shown that the larger dose was always fatal and that the smaller dose was nearly always fatal to rats of this strain.

α-Tocopherol acetate was administered intraperitoneally in a concentrated form (1 mg. = 1 international unit) or diluted with arachis oil.

Sodium selenite was administered intraperitoneally in aqueous solution. The dose of selenite was previously determined on groups of similar rats and was the maximum which could be given in the course of injections without producing acute toxic effects.

NN¹diphenyl-p-phenylenediamine (100 mg.) was dissolved in 0·4 ml. arachis oil and administered intraperitoneally.

The animals were observed for 14 days after CCl_4 had been administered.

RESULTS.

Protection against an estimated lethal dose of CCl_4.

Vitamin E. α-Tocopherol acetate was given under varying conditions of timing and dosage. The results are shown in Table 1.

TABLE 1.

Effect of vitamin E in the prevention of carbon tetrachloride poisoning.

α-Tocopherol acetate was injected intraperitoneally at the times shown in the table either as the concentrate (1 mg. = 1 international unit) or diluted 1 in 5 with arachis oil. CCl_4 was given undiluted by stomach tube. Times before or after CCl_4 are designated by − or + respectively.

	Treatment with α-tocopherol			Deaths	
No. of rats	Time (hours before or after CCl_4)	Dose (I.U.)	CCl_4 (ml./kg.)	No.	p.c.
43	—	—	4·0	42	98
15	−40	95	4·0	0	0
10	−24, −6	95	4·0	6	60
15	0	95	4·0	12	80
15	0, +5	95	4·0	13	87
18	−72, −48, −24, −6	19	4·0	13	72

In rats given 4·0 ml. CCl_4/kg., the death rate in a control group of 43 was 98 p.c. whereas in a group of 15 given 95 I.U. of the vitamin 40 hours before the CCl_4 all survived, protection thus being complete. When the vitamin was given later, 95 I.U. at 24 hours and at six hours preceding CCl_4 administration, it provided less protection as 60 p.c. of the 10 rats died. If a dose of 95 I.U. was given simultaneously with CCl_4 or reinforced with a second dose five hours later the protective effect was much lower as 80 p.c. and 87 p.c. of the rats in the respective groups of 15 died. Four smaller doses of 19 I.U. given within the 72 hours preceding CCl_4 saved only 28 p.c. of 18 rats from death even though the total dose over this period was 76 I.U.

It was shown with rats not listed in Table 1 that arachis oil, the vehicle used for vitamin administration, exercised no protective effect when given alone at comparable times and doses.

Sodium selenite. If the protection afforded against carbon tetrachloride poisoning by vitamin E is the result of its antioxidant properties, the administration of sodium selenite should also be effective (Zalkin *et al.*, 1960).

Table 2 shows a death rate of 51 p.c. in a group of 18 rats injected intraperitoneally with sodium selenite at 72, 48, 24 and 6 hours before giving 2·7 ml. CCl_4/kg., compared with that of 89 p.c. of a control group of 18 rats. This treatment with selenite therefore appeared to afford some measure of protection. The results for Group 3 in Table 2 show that selenite alone, at the dose rate used, was not lethal.

NN¹-diphenyl-p-phenylenediamine (DPPD). This antioxidant has been used as an effective substitute for vitamin E in protecting against dietary liver necrosis (Schwarz, 1958), muscular dystrophy and creatinuria (Draper and Csallany, 1958). Table 3 shows that DPPD provided good protection against death from CCl_4 poisoning.

TABLE 2.

Effect of sodium selenite in the prevention of carbon tetrachloride poisoning.

Sodium selenite was injected intraperitoneally in aqueous solution at the time before CCl_4 shown in the Table. CCl_4 was given undiluted by stomach tube.

Group	No. of rats	Treatment with Na_2SeO_3		CCl_4 (ml./kg.)	Deaths	
		Time (hours before CCl_4)	Dose (mg.)		No.	p.c.
1	18	—	—	2·7	16	89
2	18	72	0·50			
		48	0·25			
		24	0·25			
		6	0·25	2·7	10	51
3	18	72	0·50			
		48	0·25			
		24	0·25			
		6	0·25	—	0	0

TABLE 3.

Effect of NN¹-diphenyl-p-phenylenediamine in the prevention of carbon tetrachloride poisoning.

NN¹-diphenyl-p-phenylenediamine (DPPD) was injected intraperitoneally in 0·4 ml. arachis oil at the times shown in the Table. CCl_4 was given undiluted by stomach tube.

No. of rats	Treatment with DPPD		CCl_4 (ml./kg.)	Deaths	
	Time (Hours before CCl_4)	Dose (mg.)		No.	p.c.
32	—	—	4·0	32	100
19	48, 24, 0	100	4·0	0	0
11	48	100	4·0	6	55
17	0	100	4·0	10	59
12	48, 24, 0	50	4·0	8	67
11	48, 24, 0	10	4·0	7	64

All of a group of 19 rats survived when given three intraperitoneal injections of 100 mg. DPPD in 0·4 ml. arachis oil 48, 24 and 0 hours before 4·0 ml. CCl_4/kg., whereas there was no survivor among the 32 control rats used in this series of experiments given this dose of CCl_4 alone.

When a single dose of 100 mg. DPPD was given intraperitoneally 48 hours before 4·0 ml. CCl_4/kg., 55 p.c. of 11 rats died, and when given simultaneously with the CCl_4 to 17 rats, 59 p.c. died (Table 3).

The protective effect of DPPD was lower but still appreciable when smaller doses were given (Table 3). Thus, when three intraperitoneal injections of either 50 mg. or 10 mg. DPPD were given 48, 24 and 0 hours before 4·0 ml. CCl_4/kg., deaths were reduced to 67 p.c. and 64 p.c. of 12 and 11 rats in the respective groups.

Effect of antioxidants on pyridine nucleotide values of liver.

Protective effects of precursors of pyridine nucleotides against CCl_4 poisoning were found to be associated with normal E_{260} values of acid extracts of whole cell homogenates and of mitochondrial suspensions of liver (Gallagher, 1960a, b). This indicated an association with a tissue content of oxidized pyridine nucleotides which was normal (Siekevitz and Potter, 1953, 1955). Similarly, Gallagher and Rees (1960) found that effective protection against CCl_4 by the antihistamine Phenergan was associated with normal E_{260} values for liver homogenates and mitochondria.

Table 4 shows that, although none of the antioxidants prevented a departure from the normal E_{260} value of acid extracts of liver homogenates, each was associated with a value much higher than that for rats given CCl_4 alone.

TABLE 4.

Effect of antioxidants and CCl_4 on E_{260} values of acid extracts of liver homogenates.

Liver homogenates were prepared and extracted with 5 p.c. trichloroacetic acid as described in the Methods section. CCl_4 was administered by stomach tube at the dose of 4·0 ml./kg. α-Tocopherol acetate was diluted in 0·4 ml. arachis oil and injected intraperitoneally at the dose of 10 I.U. 72, 48, 24 and 6 hours before CCl_4. Arachis oil was administered in 0·5 ml. doses intraperitoneally at 72, 48, 24 and 6 hours before CCl_4. Sodium selenite was injected intraperitoneally in aqueous solution in doses of 0·5, 0·25, 0·25, and 0·25 mg. 72, 48, 24 and 6 hours respectively before CCl_4. NN^1-diphenyl-p-phenylenediamine (DPPD) was given intraperitoneally in doses of 100 mg. in 0·4 ml. arachis oil at 48, 24 and 0 hours before CCl_4. All animals were killed 20 hours after dosing with CCl_4.

Group	No. of rats	E_{260} (corrected to 5 mg. fat-free dry homogenate)		
		Range	Mean	p.c. of control E_{260}
Untreated Controls	16	0·810—1·040	0·929	100
CCl_4	16	0·281—0·483	0·364	39
α-Tocopherol + CCl_4	4	0·527—0·568	0·556	60
Arachis oil + CCl_4	4	0·304—0·404	0·346	37
Na_2SeO_3	4	0·742—0·815	0·772	83
Na_2SeO_3 + CCl_4	4	0·511—0·542	0·523	56
DPPD	4	0·857—1·000	0·911	98
DPPD + CCl_4	4	0·632—0·684	0·655	71

The administration of 4·0 ml. of CCl_4/kg. produced a decrease in the mean liver E_{260} of 16 rats to 39 p.c. of that of 16 untreated control rats of the same age, weight and sex (Table 4). Arachis oil injections alone did not affect the fall in E_{260} due to CCl_4 but Table 4 shows that the prior administration of α-tocopherol acetate, sodium selenite or NN^1-diphenyl-p-phenylenediamine offset to some degree the effect of CCl_4 and maintained 60 p.c., 56 p.c. and 71 p.c. respectively of the control values of E_{260}.

As the E_{260} values for rats which were treated with the antioxidants before CCl_4 did not remain normal, it was considered possible that the antioxidants

might lead to an alteration in the ratio of oxidized to reduced pyridine nucleotides with a higher proportion in the reduced state. Acid extraction removes only the oxidized form of pyridine nucleotides so that the E_{260} value of acid extracts does not reflect the total pyridine nucleotide concentration of a tissue unless the amount of reduced pyridine nucleotide is known also. In Table 4 it can be seen that the administration of sodium selenite alone depresses the E_{260} value in acid extracts to 83 p.c. of untreated control values; this could be due to more than the usual proportion of the total pyridine nucleotide being in the reduced state.

Reduced pyridine nucleotides are extracted from tissues by 0·1N NaOH (Glock and McLean, 1955) and have a spectral absorption maximum at 340 mμ. An estimate of the reduced pyridine nucleotide content of tissues can therefore be obtained by measuring the E_{340} of alkaline extracts (Feigelson, Williams and Elvehjem, 1950).

Extracts in 0·1N NaOH of liver homogenates were made as described in the Methods section. Table 5 shows the E_{340} values for groups of three rats treated with NN¹-diphenyl-p-phenylenediamine, CCl_4 or without treatment.

TABLE 5.

Effect of NN¹-diphenyl-p-phenylenediamine and CCl_4 on E_{340} values of alkaline extracts of liver homogenates.

Liver homogenates were prepared and extracted with 0·1 N NaOH as described in the Methods section. CCl_4 was administered by stomach tube at the dose of 4·0 ml./kg. NN¹-diphenyl-p-phenylenediamine (DPPD) was injected intraperitoneally in 100 mg. doses dissolved in 0·4 ml. arachis oil at 48, 24 and 6 hours before CCl_4. All animals were killed 20 hours after dosing the appropriate groups with CCl_4.

Group	No. of rats	E_{340} (corrected to 30 mg. fat-free dry homogenate/10 ml.)		
		Range	Mean	p.c. of control E_{340}
Untreated controls	3	0·914—0·937	0·925	100
CCl_4	3	0·444—0·468	0·458	49
DPPD	3	0·802—0·811	0·805	87
DPPD + CCl_4	3	1·016—1·026	1·020	110

Table 5 shows that intraperitoneal injection of 100 mg. DPPD in 0·4 ml. arachis oil at 48, 24 and 6 hours before 4·0 ml. CCl_4/kg. maintained higher mean values of E_{340} (110 p.c.) than in untreated control rats of the same age, weight and sex and very much higher values than in rats which received 4·0 ml. CCl_4/kg. only (49 p.c. of normal).

However, alkaline extracts of tissues contain materials which absorb light at 340 mμ other than reduced pyridine nucleotides (Feigelson *et al.*, 1950). Consequently, assay by the alcohol dehydrogenase method of the actual reduced

TABLE 6.

Effect of NN¹-diphenyl-p-phenylenediamine and CCl_4 on reduced diphosphopyridine nucleotide values of liver.

Livers were extracted and assayed for reduced diphosphopyridine nucleotide (DPNH) by the method described by Spirtes and Eichel (1954). CCl_4 was administered by stomach tube to rats lightly anaesthetized with ether at the dose of 4·0 ml./kg. NN¹-diphenyl-p-phenylenediamine (DPPD) was injected intraperitoneally in 0·4 ml. arachis oil at 48, 24 and 6 hours before CCl_4. All animals were killed 20 hours after dosing the appropriate groups with CCl_4.

Group	No. of rats	DPNH (μg./g. fresh liver)		
		Range	Mean	p.c. of control value
Untreated Controls	3	175–189	183	100
CCl_4	3	85–91	89	49
DPPD	3	210–224	219	120
DPPD + CCl_4	3	176–185	179	98

diphosphopyridine nucleotide (DPNH) content of liver homogenates in appropriately treated groups of rats was carried out.

The administration of 100 mg. DPPD in 0·4 ml. arachis oil at 48, 24 and 6 hours before 4·0 ml. CCl_4/kg. led to the retention of 98 p.c. of the mean DPNH values for normal untreated controls; whereas 4·0 ml. CCl_4/kg. alone depressed the DPNH concentration to 49 p.c. of normal (Table 6). This Table shows that DPPD given to rats without subsequent treatment with CCl_4 produced a higher concentration (120 p.c.) of DPNH than in control animals.

The effect of DPPD on liver cell necrosis.

The study of NN¹-diphenyl-p-phenylenediamine was extended to determine whether the liver was protected against the histological damage associated with CCl_4 poisoning.

Fig. 1 shows a microphotograph of a section of the liver of a rat which was killed 24 hours after receiving 4 ml. CCl_4/kg. by stomach tube. The section is typical of sections from other animals in the group and shows widespread severe centrilobular necrosis and fatty change.

Fig. 2 shows a microphotograph of a liver section from a rat which received the same dose of CCl_4 24 hours before it was killed, but which had been injected intraperitoneally with 100 mg. DPPD in arachis oil at 48, 24 and 0 hours before CCl_4 was administered. The histological appearance is almost normal without evidence of centrilobular necrosis and with very little fatty change, and is typical of liver sections of other rats similarly treated.

A single dose of 100 mg. of DPPD given 48 hours before 4 ml. CCl_4/kg. also protected liver parenchyma against necrosis as shown in Fig. 3.

Fig. 1. Section of liver stained with H. and E. Magnification X 10. Rat killed 24 hours after receiving 4 ml. CCl_4/kg. by stomach tube. The section shows widespread centrilobular necrosis and fatty change.

Fig. 2. Section of liver stained with H. and E. Magnification X 10. Rat killed 24 hours after receiving 4 ml. CCl_4/kg. by stomach tube. The animal had been given 100 mg. DPPD in 0·3 ml. arachis oil intraperitoneally at 48 hours, 24 hours and 0 hours before CCl_4. The histological appearance is almost normal.

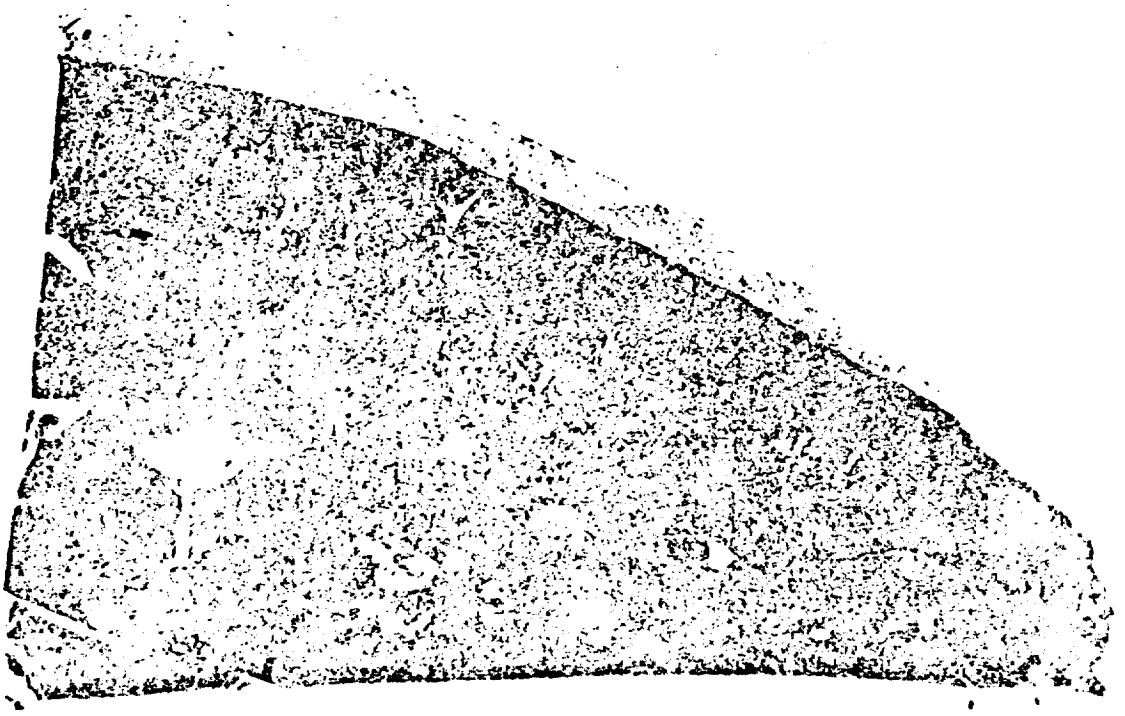

Fig. 3. Section of liver stained with H. and E. Magnification X 10. Rat killed 24 hours after receiving 4 ml. CCl_4 kg. by stomach tube. The animal had received a single dose of 100 mg. DPPD in 0·3 ml. arachis oil intraperitoneally 48 hours before CCl_4. The histological appearance is mainly normal but shows some fatty infiltration and capsular reaction to DPPD deposition.

DISCUSSION.

Assuming that the death of liver cells in animals poisoned with CCl_4 results from failure of cell respiration secondary to the loss of mitochondrial pyridine nucleotides (Christie and Judah, 1954), two lines of approach appeared feasible in searching for agents which might prevent or counter CCl_4 poisoning. One approach was to stimulate the synthesis of pyridine nucleotides in the expectation that the pyridine nucleotides lost as a result of CCl_4 poisoning would be so rapidly replaced by fresh synthesis that the mitochondria would continue to respire. This expectation was realised with the prior administration of nicotinic acid or tryptophane which are precursors of pyridine nucleotides (Gallagher, 1960a, b). The other approach was to prevent the loss of pyridine nucleotides from mitochondria despite the administration of CCl_4.

It was found in the present studies with rats that α-tocopherol acetate, sodium selenite and NN¹-diphenyl-p-phenylenediamine each afforded some protection against the lethal effect of large doses of CCl_4. These three substances have antioxidant properties in common and can be used as substitutes for each other for some biological purposes, for example, in countering such clinical manifestations of vitamin E deficiency in animals as liver necrosis, creatinuria and muscular dystrophy (Bunyan, Edwin and Green, 1958; Schwarz, 1958; Zalkin *et al.*, 1960).

Under the conditions of the present study, NN¹-diphenyl-p-phenylenediamine appeared to be the most effective of the three antioxidants tested in increasing the survival rate in CCl_4 poisoning. Both DPPD and α-tocopherol, when given two days previously, protected all rats against a lethal dose of CCl_4. However, a single dose of DPPD given simultaneously with a lethal dose of CCl_4 afforded a greater measure of protection than a single large dose of vitamin E given with CCl_4, or even this supplemented five hours later with the same large dose of vitamin E. Complete protection was not obtained with sodium selenite against smaller doses of CCl_4.

It was considered that the antioxidants probably acted by preventing the series of reactions which results in the loss of mitochondrial and cellular pyridine nucleotides subsequent to the primary damaging effect of CCl_4. Gallagher (1960c) showed that ethylenediaminetetraacetic acid does this *in vitro*. In order to determine whether the antioxidants were acting similarly *in vivo* the effect of their prior administration on the pyridine nucleotide content of liver was investigated.

In the case of pyridine nucleotide precursors Gallagher (1960a, b) found that prior administration led to the maintenance of normal levels of oxidized pyridine nucleotides in liver despite very large doses of CCl_4.

The administration of antioxidants before CCl_4 preserved much higher levels of oxidized pyridine nucleotides, as indicated by E_{260} values of acid extracts of liver homogenates, than in rats given the same dose of CCl_4 alone but the E_{260} values maintained by the use of antioxidants were below normal values for rats of the same age, sex and weight.

Depression of the concentration of oxidized pyridine nucleotides could be due either to their loss from cells or to alteration of the ratio of oxidized to reduced pyridine nucleotides with a higher proportion in the reduced state. As antioxidants might conceivably alter the balance of oxidized and reduced pyridine nucleotides, the reduced pyridine nucleotide content of livers from rats treated with DPPD and CCl_4 was determined.

An estimate of the effect of CCl_4 and DPPD on reduced pyridine nucleotides was obtained by extracting homogenates of liver with 0·1N NaOH and measuring the E_{340} of the extracts. It was clear that CCl_4 alone led to a large loss from liver of material absorbing light at 340 mμ (49 p.c.) and that this

loss was completely prevented by prior treatment with DPPD. The E_{340} values maintained by DPPD despite CCl_4 administration were, in fact, higher (110 p.c.) than those for control untreated rats (100 p.c.).

Liver contains substances which absorb light at 340 mμ other than reduced pyridine nucleotides. It was important therefore to measure the actual amount of reduced pyridine nucleotide present. Reduced diphosphopyridine nucleotide (DPNH) was measured by the alcohol dehydrogenase method. The results showed that rats treated with DPPD maintained normal levels of DPNH despite subsequent administration of CCl_4. CCl_4 alone reduced the concentration of DPNH to 49 p.c. of the value for untreated control rats. The administration of DPPD alone led to higher than normal DPNH values (120 p.c.).

Prior administration of DPPD to rats afforded considerable protection against the liver damage consequent to a large dose of CCl_4. Cell necrosis was prevented, and the fatty change to a considerable extent, by the antioxidant.

This study supports the hypothesis initially put forward by Christie and Judah (1954) that liver cell death in CCl_4 poisoning is due to the loss of pyridine nucleotides from mitochondria and the consequent failure of respiration. The antioxidants, in preventing the death of rats and of liver cells when given prior to a lethal dose of CCl_4, maintained at least normal levels of reduced pyridine nucleotides in the liver and much higher levels of oxidized pyridine nucleotides than those resulting from CCl_4 alone. The argument that the loss of pyridine nucleotides may follow cell death and that antioxidants prevent the loss of pyridine nucleotides by preventing cell death does not seem tenable because it has been found that the concentration of oxidized pyridine nucleotides in the liver is decreased within 1-3 hours of administering CCl_4, long before the usual time of onset of liver cell necrosis (Gallagher, 1961).

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Obligatory Role of Bile for the Intestinal Absorption of Vitamin E

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ABSTRACT

Normal, white female rats subjected to cannulation of the abdominal thoracic duct have been utilized for a study on the essentiality of biliary and pancreatic secretions for the intestinal absorption of vitamin E. In all animals the thoracic duct lymph was collected. Some rats had the enterohepatic circulation undisturbed and in others bile or pancreatic juice or both were drained to the exterior by appropriate catheters in the common bile duct. On the first postoperative day, the animals received intragastrically an emulsion containing protein, carbohydrate, monoolein, 2 mg of *d,l*-α-tocopheryl acetate plus 50 μC of *d,l*-α-tocopheryl-1',2'-^{3}H-acetate. The appearance of radioactive α-tocopherol and its derivatives was determined in lymph, hourly, after emulsion administration. The obligatory role of bile in intestinal absorption of *d,l*-α-tocopheryl-1',2'-^{3}H-acetate has been established. Pancreatic juice seems to be necessary for the hydrolysis of the vitamin E acetate ester. The simultaneous infusion of bile and pancreatic juice promotes absorption of about 10% of the administered dose into the lymph. A chromatographic separation of the radioactive vitamin E fractions revealed that most of the vitamin E, which is actively transferred from the intestinal lumen to the lymph, is nonesterified. An oxidation product of α-tocopherol, presumably its *p*-quinone, appears in small amounts in the lymph, but almost no labeled α-tocopheryl acetate could be detected under these experimental conditions.

INTRODUCTION

Although it has been recognized for many years that bile salts and pancreatic secretions affect the absorption of dietary vitamin E (1,2) evidence on this matter has been incomplete (3). Infants with congenital atresia of the bile ducts or with cystic fibrosis of the pancreas have diminished concentrations of plasma tocopherol and creatinuria and show an abnormal erythrocyte hemolysis test. These defects are presumably due to poor absorption of the vitamin in the absence of biliary and pancreatic secretions from the intestine. However, this interrelationship has not been clearly substantiated.

Most studies made in animals and humans have employed either metabolic balance techniques estimating tocopherol absorption from measurements of tocopherol intake and fecal or urinary excretion (4-8) or measurements of changes in the vitamin levels of plasma, liver and other organs (9-15). Although these studies have provided excellent information about the distribution of small amounts of vitamin E and its metabolites in the body, the use of such techniques makes interpretation of the absorption data difficult.

Direct evidence for the absorption of ^{14}C-α-tocopherol into the intestinal lymph of rats was reported by Johnson and Pover (16). Since in that study samples of lymph were pooled over variable periods of time from 27 to 96 hr after feeding of the labeled vitamin, observations on the kinetics of absorption during the postabsorptive period were precluded. Blomstrand and Forsgren (17) have recently reported on the intestinal absorption of several labeled tocopherols in man. However, no complete study on the effect of biliary or pancreatic secretions or both on vitamin E absorption is available in the literature.

It was, therefore, considered important to carry out a study on the influence of biliary or pancreatic secretions or both on the intestinal absorption of vitamin E. This investigation was facilitated by the recent development of a new technique for the cannulation of the abdominal thoracic duct in the rat (18) and the availability of *d,l*-α-tocopheryl-1',2'-^{3}H-acetate. In addition, the total radioactivity and the vitamin E radioactive fractions of lymph were determined hourly in an attempt to obtain a more detailed picture on the kinetics of absorption of vitamin E in the presence or absence of bile.

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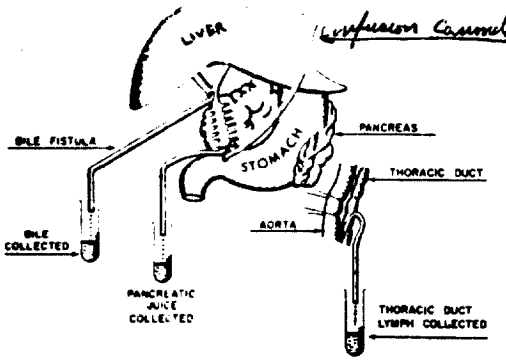

FIG. 1. Thoracic duct cannula and triple catheterization of the common bile duct in the rat. This preparation was used in some of the studies reported here.

MATERIALS AND METHODS

Female albino rats (300-350 g) of the CD Charles River Breeding Laboratories (North Wilmington, Mass.) were maintained on a standard Purina chow diet and water ad lib until the time of operation.

Surgery

The detailed procedure for the cannulation of the abdominal thoracic duct in the rat has appeared (18). In some instances, rats with thoracic duct fistula but with reconstituted enterohepatic circulation were used. In other instances, the animals had a lymph fistula plus two catheters in the upper part of the common bile duct; through the distal catheter, the infusion cannula, 3.6 ml of saline (0.85% NaCl plus 0.04% KCl) were infused. A third preparation used in the present studies consisted of a thoracic duct fistula plus three catheters in the common bile duct (Fig. 1). Use of this preparation permitted the infusion into the duodenum of either saline alone (duodenum devoid of bile and pancreatic secretions) or saline plus bile uncontaminated with pancreatic juice; in both instances the animal's own bile and pancreatic juice were diverted from the intestine. After operation, the animals were placed in a restraining cage (19) made by C. H. Stoelting Co., Chicago, Ill.

EXPERIMENTAL PROCEDURES

Absorption experiments began 18 to 24 hr after operation. According to the protocol for the studies of cholesterol absorption (20) the detailed composition of the emulsion used has been measured elsewhere (20) [illegible] consisted basically of 50 μC (140 μg) of pure d,l-α-tocopheryl-1',2'-^{3}H acetate (see below), protein, carbohydrate, monoolein, saline and 2 mg of *d,l*-α-tocopheryl acetate in 50 μl ethanol. Both the carrier and the labeled vitamin E ester were added to tubes containing 4 ml of the emulsion and mixed by means of a Vortex Jr. Mixer (Model K-J, Scientific Industries, Inc.). Animals were given the emulsion by stomach intubation without the aid of anesthesia. Lymph was collected at hourly intervals for 12 hr. Bile samples were also collected along with the lymph in the cases of animals with common bile duct catheterizations.

Purification of Radioactive Material

D,l-α-tocopheryl-1',2'-^{3}H-acetate (F. Hoffmann-La Roche & Company, Ltd., Basle, Switzerland) had a specific activity of 356 μC/mg in 1962. By using glass fiber paper chromatography techniques described below, it was found that the original material contained breakdown products of α-tocopherol, and, therefore, it was purified by passing it through a 15 x 3.5 cm column of silicic acid (100 mesh, Mallinckrodt). After activation and packing, the column was first washed with 400 ml of *n*-hexane (Chromatoquality Reagent, 99 + mole %, Matheson Coleman & Bell, East Rutherford, N.J.); 90 mg of the tritiated α-tocopheryl acetate were dissolved in 5 ml *n*-hexane and poured into the column. Mixtures of *n*-hexane-benzene (100:5 up to 100:50) were used to successively wash the column. Benzene (Reagent, A.C.S., Lehigh Valley Chemical Co., Easton, Pa.) was distilled before use and kept dry by the addition of calcium chloride to the bottle. Eluents of each solvent mixture were collected in 25 ml fractions; 5 μl aliquots were spotted in small portions on glass fiber paper and the radioactivity counted in a Packard Scintillation Spectrometer by procedures similar to those described for the counting of cholesterol and its esters (20). Most of the radioactivity was found in the eluates consisting of a mixture of hexane-benzene, (100:35). Final purity of the *d,l*-α-tocopherol ester was checked by glass fiber chromatography immediately prior to use. Using suitable standards of free α-tocopherol and *d,l*-α-tocopheryl acetate (both from Hoffmann-La Roche Inc., Nutley, New Jersey), it was found that the fraction corresponding to the radioactive tocopheryl acetate was more than 99% pure.

Vitamin E Extraction Procedures

Lymph samples were extracted immediately after collection. The method used for the

extraction of lipids from lymph is reported in detail elsewhere (20). Briefly, this procedure involved extraction of the lymph with ethanol-isopropyl ether (2:1 v/v) and in the ratio of 1 ml of lymph per 20 ml of extracting mixture. After centrifugation, the supernatant was dried under N_2 and the samples were made up to a known volume (0.3 to 1.0 ml) with *n*-hexane, and aliquots of 100 μl used for chromatography. Absolute ethanol (U.S.P. 200 Proof, Distilled by Publicker Industries, Inc., Philadelphia, Pa.) was freed of aldehydes by redistillation over $KMnO_4$ (Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.) and KOH pellets (Fisher Scientific Co., Fair Lawn, N.J.) and kept in dark bottles until use. Isopropyl ether (Practical, Matheson Coleman & Bell, East Rutherford, N.J.) was freed of peroxides by procedures reported previously (20). The lymph extraction mixture was prepared immediately after purification of isopropyl ether.

Chromatography and Radioactivity Measurements

The separation of free tocopherol from tocopheryl acetate and from other lipid components was carried out by glass paper chromatography using glass fiber paper obtained from Gelman Instruments Co., Ann Arbor, Michigan (ITLC-SG) and employing two solvent systems. The first solvent system consisting of isooctane-benzene (100:15 v/v) is the same used for the separation of cholesterol and cholesterol esters in blood (21) and lymph (20). Using this system, free tocopherol traveled just below cholesteryl arachidonate and separated quite well from α-tocopheryl acetate, which traveled much lower than the unesterified vitamin. Oxidation products of α-tocopheryl remained at the origin of the chromatogram. These oxidation products were chromatographed by using an acidic solvent system consisting of isooctane-ethyl ether-formic acid (100:10:0.5). Anhydrous ethyl ether (Reagent A.C.S.) and formic acid (both purchased from Allied Chemical, Morristown, N.J.) were used without further purification. Using this solvent system, free tocopherol and its acetate ester traveled close to the solvent front. The oxidation product comparable to the one obtained after reaction of α-tocopherol with $FeCl_3$ in absolute ethanol, presumably the α-tocopheryl-*p*-quinone (22), remained distinctly isolated in the middle of the chromatogram.

Glass fiber paper chromatography of the lymph extracts was done in duplicate and excellent agreement was obtained. Suitable mixtures of standards were applied to one side of the chromatograms as 5 μl (0.4 μg) aliquots; after developing and drying of the chromatogram, the side (about 3 cm wide) containing the mixtures of standards was cut, sprayed with concentrated sulfuric acid and charred over a hot plate. The corresponding radioactive bands were systematically removed and prepared for counting as described (20). The counting was carried out in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3380, with Model 544 Absolute Activity Analyzer attachment. The results are thus expressed in dpm per hour of lymph production.

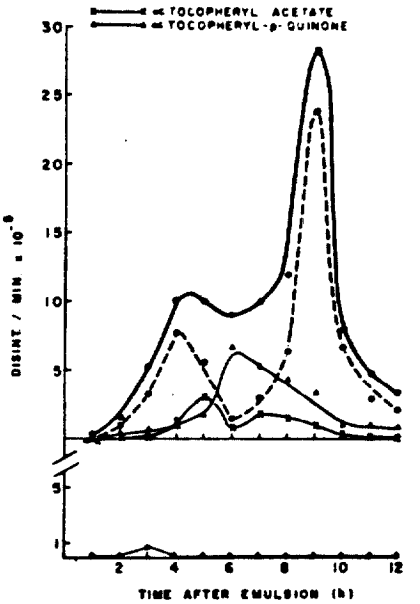

FIG. 2. The appearance of radioactive α-tocopherol and derivatives in the thoracic duct lymph of rats at hourly intervals in the presence of biliary and pancreatic secretions (upper graph) or in their absence (lower graph). In both instances, an emulsion was administered containing protein, carbohydrate, monoolein and tritiated α-tocopheryl acetate. Each graph is representative of three experiments.

RESULTS

Simultaneous Effect of Bile and Pancreatic Juice on Tocopherol Absorption

Three animals were prepared in which a thoracic duct fistula was performed but had the enterohepatic circulation reconstituted by an external shunt. Saline was given ad lib. The emulsion, containing α-tocopheryl-1',2'-$^3H_2$-acetate was given 20 hr after operation. The appearance of radioactive α-tocopherol and its derivatives in lymph is shown in Figure 2. Two

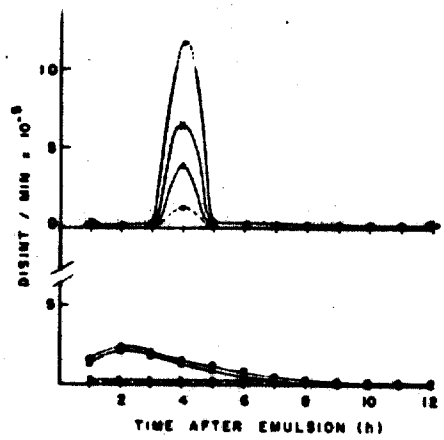

FIG. 3. The appearance of radioactive α-tocopherol and derivatives in the thoracic duct lymph of rats when either pancreatic juice (upper graph) or bile (lower graph) is present in the intestine. Other experimental conditions as described in Figure 2. Each graph is representative of three experiments.

distinctive radioactivity peaks are seen, an early peak, occurring at 3 or 4 hr after feeding and a second, more significant peak, occurring 6 to 9 hr after administration of the emulsion. Most of the radioactivity was found in the form of free tocopherol; however, there was some in the form of an oxidized product, presumably α-tocopheryl-*p*-quinone and a very small amount in the fraction corresponding to the tocopheryl acetate ester. About 10% of the administered dose of radioactivity was recovered in the 12 hr following the intragastric administration of the emulsion under these experimental conditions.

In a second group of experiments, three animals were prepared in which a thoracic duct was created and both biliary and pancreatic secretions drained to the exterior by means of a cannula in the lower part of the common bile duct, through the same opening on the bile duct, another catheter was inserted and introduced 2-3 mm into the intestine. This cannula was used to infuse saline (3.6 ml hr) into the duodenum. Negligible amounts of radioactivity were detected in the thoracic duct lymph of the rats under these circumstances (Fig. 2). There was no detectable peak absorption in any of the three experiments and the amount of radioactivity appearing in the lymph was 0.1% of the administered dose (on the average) during the 12 hr after feeding. At this extremely poor level of absorption, there was a predominance of the oxidized form of the tocopherol and virtually no free tocopherol appeared in lymph during this period.

The effects of the Infusion of Pancreatic Juice Or Bile Alone on Tocopherol Absorption

The previous experiments showed that both pancreatic and biliary secretions, when given simultaneously, promote a considerable enhancement of the absorption of vitamin E from the intestine of the rats. It was decided, therefore, to study the effect, separately, of each of these two secretions.

Three rats were used in which bile was drained to the exterior, pancreatic juice left undisturbed, and a thoracic duct fistula created. The animals were hydrated as described in the Materials and Methods section. Figure 3 shows that, after administration of the radioactive α-tocopheryl-acetate-containing emulsion, the absorption rate of tocopheryl is very much reduced owing to the lack of bile. An average of 1.5% of the total radioactivity administered was recovered in the 12 hr following feeding. There was an absorption peak by the third or fourth hour. Most of the radioactivity found in lymph was due to the labeled α-tocopheryl acetate and significant amounts were found in the oxidized form. Radioactive free tocopherol was either absent or very low during the 12 hr of lymph collection.

In a last group of experiments, the effect on tocopherol absorption of infusion of bile alone into the duodenum was studied. For this purpose, bile, uncontaminated with pancreatic juice (18), was collected for up to one week from several rats and the pooled collections kept at 4 C until use. Three animals were cannulated in the thoracic duct and thrice cannulated in the common bile duct (Fig. 1). The catheter in the upper part of the common bile duct drained pure bile; one of the two catheters in the lower part of the common duct was used to drain pure pancreatic juice from the animal's body. Through the infusion cannula, each rat received 1.5 ml bile (from experimental data on bile flow in these well hydrated fistulated animals, it has been found that from 0.8 to 1.5 ml hr of bile is produced) plus 2.1 ml saline per hour into the intestine. Owing to the lack of pancreatic juice, a severely reduced rate of absorption was observed in lymph during the 12 hr following administration of the emulsion containing radioactive α-tocopheryl acetate (Fig. 3). It was seen that there is no real peak absorption; total radioactivity was somewhat higher during the first 5 hr after feeding, and about 1.3% of the administered radioactive dose was recovered in the 12 hr lymph collection. As in the previous instances of poor absorption, the fractions of vitamin E corresponding to the oxidized form and to tocopheryl acetate had most of the radioactivity

and very little, if any, free tocopherol was found.

DISCUSSION

The studies reported here are the first in which the appearance of radioactive tocopherol fractions has been followed as a function of time during conditions of presence and absence of bile in the intestine. This was accomplished after feeding identical amounts of the radioactive *d,l*-α-tocopheryl acetate in an emulsion. Since the same concentration of the vitamin E acetate ester was administered in each instance, it was possible to carry out appropriate comparisons under the several conditions studied. Infusion of saline at a constant rate has assured not only an adequate hydration, but a uniform lymph flow as well (18). The results of this study have provided definitive evidence that the presence of bile salts and pancreatic juice in the intestine is a requisite for the intestinal absorption and the lymphatic transport of vitamin E.

During the simultaneous infusion of bile and pancreatic juice into the duodenum, labeled vitamin E appears in lymph mostly as the unesterified tocopherol and very little as the tocopherol ester. Two peaks of radioactivity appear and this biphasic type of kinetics suggests the existence of a dual mechanism of absorption. Data obtained indicate that the infusion of pancreatic juice alone can support appearance of vitamin E in lymph, which, quantitatively, is very similar to that seen in the early peak of absorption under conditions of reconstituted enterohepatic circulation. However, qualitative differences were evident and more experiments would be necessary to further clarify this observation.

The recovery of 10% of the radioactivity from the administered dose agrees with that reported by Simon et al. (6) after feeding *d*-α-tocopheryl-5-methyl-^{14}C-succinate to rabbits. However, in the present study, unlike that of Simon et al. (6), no recirculation of the absorbed isotope to the intestine is possible because the absorbed labeled vitamin E is diverted outside the animal's body owing to the lymph fistula. Since data reported herein were obtained under ideal conditions for absorption, i.e., normal animals with undisturbed enterohepatic circulation to which a physiological dose of tocopheryl acetate (2.14 mg) was administered in a fat-containing emulsion, it can be concluded that α-tocopheryl acetate is a fat-soluble ester which is poorly absorbed from the intestinal tract, even though this compound is liquid at room temperature and presumably might be soluble in micellar bile salt solutions.

The poor absorption of the vitamin E acetate ester is in sharp contrast with that of other lipid materials, notably, glycerides, which are absorbed, after intestinal digestion, with an efficiency close to 100%. This observed dissimilarity is perhaps indicative of a lack of parallelism between vitamin E and glycerides during digestion, intestinal absorption and lymphatic transport. From the above considerations, it is also clear that absorption data, based on fecal excretion of a substance or its metabolites after feeding of the radioactive compound, should be interpreted with caution, especially if the compound, as in the case of vitamin E (23), undergoes an enterohepatic circulation. Recirculation of the absorbed radioactive material might possibly affect the fecal excretion data.

The appearance of radioactive vitamin E in lymph was at a minimum in the absence of either bile or pancreatic juice. Two main radioactive fractions are detected in lymph under these very poor absorption conditions: the α-tocopheryl-*p*-quinone and traces of the administered ester; unesterified vitamin E is almost completely absent under these conditions. These data suggest that the small quantity of radioactive vitamin E that appears in lymph in the presence of only pancreatic juice in the lumen is the result of an entirely different and inefficient mechanism than the one operating when both bile and pancreatic juice are present in the duodenum. These findings clearly emphasize the obligatory role of biliary secretions in the absorption of α-tocopheryl acetate. They also explain why vitamin E deficiency syndrome develops under conditions in which there is insufficient bile or pancreatic juice in the gastrointestinal tract (1,2).

The requirement for bile indicates that emulsification of this water-insoluble vitamin is required prior to its penetration into the intestinal mucosa. Pancreatic juice presumably might contain an enzyme (α-tocopherol ester hydrolase) the existence of which, as well as its site of action (intraluminal, surface or interior of the intestinal cell), remains to be demonstrated. Indeed, from the present studies, it is evident that in the rat the first step in the absorption of α-tocopherol ester is hydrolysis. In those cases of good absorption, there was extensive hydrolysis of the administered α-tocopheryl acetate, which presumably occurred in the intestinal lumen, and accounts for the virtual absence of tocopheryl acetate in the lymph. This absence of vitamin E ester in the lymph of the rat is in complete agreement with reported data on the absorption and distribution of tocopheryl acetate in chicks (8). Aside from its

In summary, we note:

1. A protective effect on adrenalin by ascorbic acid [2] and [4];
2. a potentializer (strengthening?) effect in vitro [7, 21, 26] and in vivo [1],13,]0[of the vitamin P factors on the adrenalin, hence prolonging of the sympathomimetic action;
3. a protective action of ascorbic acid by the vitamin P factors.

The chromanic structure common to catechine and vitamin E led me to seek out whether the d-1-alpha tocopherol can exert, in its turn, effects which are comparable to those of the P factors.

The antioxygen properties in vitro of the d-1-alpha-tocopherol are well known, but Dubouloz et al. []0] noted that this substance can also be replaced in vitro by other antioxygens in the favorable action exerted by these products on the putting into reserve of vitamin A into the liver. Vogelsang et al., [25] as well as Barczi [3] noted the improvement brought about by tocopherol in the capillary circulation and in the utilization of oxygen by the tissues. Ratcliffe [22] believes that this product should favor the proliferation of new capillaries from the good results which he obtained in the intermittent claudication. Herve and Bacq []]], lastly, noted a reduction in mortality in mice exposed to X rays and treated with tocopherol.

All of these observations seem to pose the following problems:

1. What is the action of the d-1-alpha-tocopherol alone on the histamine bronchospasm?

2. What is its action, in association with other vitamins, on this bronchospasm?

3. What capillary effect does it supply alone or in association with other vitamins?

In this paper I will attempt to answer the first one of these questions.

Method: Guinea pigs were distributed into six batches of 5 animals grouped so as to comply with two requirements: weight about equivalent, same sensitivity to histamine. The latter test was obtained by placing each guinea pig into an aerosol engendered by the pulverisation of an aqueous glucerinated solution of bichlorhydrate of histamine (at 4 per 1,000) . The sensitivity to the histamine is the time taken by each animal to fall over on its back. Since the variations in the times inside of each batch or lot were of a small degree (three to five seconds more or less) I have reproduced in the tables below only the mean of the five times noted.

Per day the animals of the first batches received in a single time, respectively, 25 mg and 5 mg of acetate of d-1 alpha-tocopherol in intraperitoneal injection. The animals of the third batch received 10 mg per dat of the same substance "through the mouth" (per os). Those of the fourth batch bring the control figures concerning the histamine alone. Lastly those of the fifth and six batches show the action of a solution very concentrated in histamine (4 percent instead

of 4 per thousand , i.e. 4% instead of 4 per mil) according to whether or not the guinea pigs received for seven days 1.25 mg of d-l-alpha-tocopherol per os and per day. Each one of the animals of these last two batches were subjected every day for seven days for twenty seconds tothe action of the aerosol. This time, not enough to cause an attack of asthma, was intended to correct the slight sensitizing action due to the histamine during the first applications (see table 4).

The first action of the histamine on all of the animals only occurred after the second ingestion or digestion. Each histamine test started one hour after the injection or three hours after the ingestion.

First batch: 25 mg of d-l-alpha-tocopherol per animal and per day by intraperitoneal injection.

table p. 205 key:

1= days; 2= average weight; 3= time necessary to lose balance
4= observations 5= death of all of the animals

JOURS	POIDS MOYENS	TEMPS NÉCESSAIRES à la perte de l'équilibre	OBSERVATIONS
1	505 g.		
2	—	1 min.,57 sec.	
3	—	1 min.,02 sec.	Mort de tous les animaux.

Second batch: 5 gg or d-l-alpha-tocopherol per animal and per day by intraperitoneal injection.

key :
1= days, 2= average weight, 3= time necessary to lose balance,
4= observatoions, 5- survival of all of the animals

Third batch: 10 mg of d-l-alpha-tocopherol, per os, per animal and per day.

key: 1= days, 2= average weight, 3= time necessary to lose balance,
4= observations, 5= survival of all of the animals

JOURS	POIDS MOYENS	TEMPS NÉCESSAIRES à la perte de l'équilibre	OBSERVATIONS
1	440 g.		
2	—	2 min.,7 sec.	
3	—	1 min.,50 sec.	
. . . . .	440 g.	1 min.,40 sec.	
. . . . .	—	1 min.,46 sec.	
. . . . .	—	1 min.,57 sec.	
. . . . .	405 g.	2 min.,15 sec.	Survie de tous les animaux.

II

JOURS	POIDS MOYENS	TEMPS NÉCESSAIRES à la perte de l'équilibre	OBSERVATIONS
1	375 g.		
2	—	1 min.,32 sec.	
3	—	1 min.,50 sec.	
4	380 g.	1 min.,40 sec.	
5	—	1 min.,19 sec.	
6	—	1 min.,45 sec.	
7	382 g.	2 min.,02 sec.	Survie de

III

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CAN d-1 ALPHA-TOCOPHEROL EXERT A PROTECTIVE ACTION ON THE ADRENALIN OF THE TISSUES IN THE SAME WAY AS THE VITAMIN P FACTORS?

by M. G. Gazet du Chatelier *

In the last few years numerous vitamins have revealed a rather significant antihistamine action. Among the authors who have attached their names to this type of research let us mention Balakhovskij et al. [1] (vitamin A); Lovino and Lecco [17] (vitamine B_2); Lecoq, Chauchard add Mrs. Mazoue [15, 16] (vitamins D, C and P factors); Businco [5] (vitamins C and PP); Castelli and Destefanis [6] (vitamine PP); Traina [23] (vitamin B_{12}).

The interest of these studies seems indisputable and therapy may derive considerable profit from this.

A noteworthy phenomenon in the action of certain of these vitamins (in particular the P factors and vitamin C) concerns their metabolism, in comparison to that of adrenalin, the vaso-constrictive and bronchodilating effects of which could be directly related to the antihistamine effect attributed to vitamins. Castelli and Destefanis already pointed out that the role played by nicotamide should be related with its high oxide-reducing potential. But Babac and Roseman [2] gave the proof the stabilization of certain polyphenols in an alkaline medium thanks to ascorbic acid and cystein. Beauvillain and Sarradin [4] confirmed this work in showing that "ascorbic acid plays the dual role of protecting the ortho-diphenol groupina dnd preventing the cyclisation of the lateral chain by the appearance of the ketone function during the course of spontaneous oxidation to air". Now precisely, similar phenomena were observed in vivo for the vitamin P action. Thus Lavollay [12] noted that after catechine injection there was a "considerable increase in the capillary resistance of the Cobaye* as well as an increase in the duration of action of the adrenalin on the nictitating membrane of the cat". Subsequently other experiments by Lavollay in conjunction with Parrot [20] confirmed these initial results. The phenomena is to be attributed, according to Lavollay, to an inhibition of the autooxidation of the adrenalin. This author adds, very pertinently [12]: "One will note that as another vitamin, vitamine E, this substances(catechins) possesses the chromanic structure".

In their turn, Clark and Geissman [7] on the one hand, Wilson and DeEds [26] on the other hand, found a "potentialization of the effects of adrenalin by vitamin P" (a strengthening) and the flavonoids in general. Cotereau [8], furthermore, found a correlation between vitamin C and the P factors, revealing that "certain factors capable of catalysing the destruction by autooxidation of the ascorbic acid also have the property of reducing the capillary resistance" and that the "derivatives of flavone, capable of raising the capillary resistance, as well as a close derivative particularly active in this biological test, epicatechine, were all provided with the property of slowing down, in vitro, the oxidation of the ascorbic acid by the oxygen of the atmosphere".

* Cobaye = French term for cavy or Guinea pig.

emulsifying properties, the presence of bile salts could be a requisite for the hydrolysis of α-tocopherol acetate prior or during penetration of vitamin E into the mucosal cell. It was demonstrated by Greaves and Schmidt (1) that the oral administration of deoxycholic acid increases the absorption of vitamin E in bile-fistulated rats. Thus, these authors provided indirect evidence that an individual bile acid is able to promote the absorption of vitamin E. It would, therefore, be of interest to demonstrate whether free or conjugated individual bile acids promote the absorption of unesterified or esterified vitamin E or both at different rates into the thoracic duct lymph.

ACKNOWLEDGMENTS

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to prevent sterility; and a dose equal to $\frac{N}{10}$ would permit the formation of useless reserves for the experiment. According to the author, a single dose of 1.5 mg. of vitamin is sufficient to maintain the fertility of the female rat. Guided by those precedents, it was considered that a dose of 0.6 mg daily of Vitamin E equal to $\frac{40N}{100}$ would be sufficient to maintain the fertility of the male rat although the male requirement of vitamin E is more that that of the female (Mason 1933, Evans 1938, Evans and others 1939). At this time we don't have an experiment that gives data oriented toward establishing the requirements of vitamin E in the male. Our groups were established to receive a small supplement of vitamin E everytime. The last group received a dose 100 times smaller than the first.

Forms of Administration of Vitamin E Supplements: The vitamin E was administered in a suspension of starch without dextrins. This suspension was handled in the same manner as the product absorption standard for vitamin B_1.

Methods Used in the Reading of the Results of This Experiment: Different methods were employed in determining the fertility of the animals used in the different groups.

1. Mating the animals during the period of seven days with females of proven fertility and determining by autopsy they were able to produce young. In the first case, a number of animals were contained in the uterus.

2. Determining the weight of the testacles of the animals in different groups.

3. Determining byhistological study the state of the testicles of the animals.

CLINICAL STUDY. In man the action of the d-l-alpha-tocopherol seems to be of greater interest, at least in the very precise case of persons suffering from hay fever. In effect, at the daily dose of 10 to 15 mg, this substance exerts a preventive and curative action. If the hay fever has set in quite firmly we see the symptoms (in the eyes, ears, bronchi, etc.) regress and disappear in thirty six or forty eight hours. Only the rhinorrhea (watery discharge from the nose) retrocedes sometimes incompletely. However, the nasal passages are completely freed and disobstructed by adding to tocopherol 0.10 g of ascorbic acid.

INTERPRETATION OF THE RESULTS

Independently of the antitoxic action exerted by the d-l-alpha tocopherol at weak dose [2 mg 50/kilo approximately] (2) vis a vis a concentrated solution of histamine (an action which is perhaps not exactly superimposable to a conventional antihistamine action) can we actually speak of disagreement between the negative results contained in tables 2 and 3 and the positive action observed in man in hay fever (3)? To do this it would naturally be necessary to assimilate, as to their causes, a histamine brochnospasm to hayfever. Nothing, however, permits us to attribute at the present time to histamine any determining action in this ailment. To speak of disagreement one would also have to be able to prove that a substance cannot have in vitro, an anti-allergic action when it does not produce in the animal a clearly appreciable action on the histamine brochospasm. But it is known precisely that the anaphylactic shocks can be attenuated, if not completely eliminated, by narcosis; ether associated with narconumal (Diedey) [9] or ether alone (Muller) [19].

Diedey does not believe in an antiallergeic effect of the narcosis due to an antihistamine effect. He observes that the narcosis does not seem to prevent the antigen-antibody reaction.

The facts reported on sparteine are also interesting in this regard. For Capretti and Guarnieri [5 bis] this alkaloid protects the dog against bronchospasm due to the inhaling of histamine aerosol, but not against the histamine shock and the vascular reactions caused by this toxic (poison). In contrast, according to Mercier [18] sparteine would resist in the guinea pig the anaphylactic shock although I was able to establish that this substance (at the dose of 5 to 8 mg/kilo) did not protect the guinea pig against histamine bronchospasm.

As we can realize from these few examples, the relationships between an experimental antihistamine action and a purely allergic phenomenon are not always easy to establish. Diedey even goes fuether and supposes that the theory according to which the allergic reactions are due to the release of histamine seem too simple.

According to what has been stated previously we could therefore deduce that a lack of protection of the guinea pig by the d-l-alpha-tocopherol against histamine bronchospasm does not permit excluding a priori a positive action of this substance in man as concerns hay fever. The pathology of this ailment brings to light an excessive sensitivity of the nasal mucous membranes , the ocular and bronchitic and other membranes. The d-l-alpha-tocopherol alone or in the presence of ascorbic acid exerts perhaps a tonic and specific action on the mucosa and their vessels, an action which might result from a prolonged effect of adrenaline due to a protection of this hormone against a too rapid oxidation (in the manner of a vitamin P action). This action would turn out to be sufficient when it is a question of preventing the occurrence of weak physico-chemical disturbances , but clearly insufficient to prevent an experimental histamine bronchospasm.

SUMMARY. The d-l-alpha-tocopherol at high dosage (80 mg/kg) sensitizes the guinea pig to the action of the histamine , causing the death of the animal irremediably after a second intoxication session. At a smaller dosage the sensitization is nil. Nevertheless even after several days of experiment this vitamin turns out to be not capable of prolonging the time taken by the guinea pig to enter into an asphyxia coma. On the other hand it protects the life of this animal during the course of a coma due to an aerosol very concentrated in histamine.

In man the d-l-alpha-tocopherol alone and above all in association with ascorbic acid seems to exert a specific action on the various symptoms of hay fever. To a certain extent we could attribute this action to that reported on in other cases as being attributed to the protective action exercised on adrenalin of the tissues by the P vitamin factors.

(School of Pharmacy of Strasbourg, Laboratory of Pharmacodynamic

key to the bibliography:

reference 8:

1= Contribution to the study of the relationships between ascorbic acid and vitamin C_2 (vitamine P) . Doctoral thesis.

reference 13

2= The autoxidation of diphenols and in particular of adrenalin. The structure and functional role of vitamin P.

reference 18:

3= Sparteine in therapy.

reference 21:

4= Introduction to the study of vitamin P.

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Distribution of radioactivity in the rat after administration of ^{14}C-dl-alpha-tocopherol acetate

by W. Gloor, F. Weber, J. Wursch, and O. Wiss

For the judgement of the mechanism of effect of vitamin E in the animal organism, a reliable knowledge of the level of alpha-tocopherol in the tissues is important. In connection with the partial replaceability of vitamin E with synthetic antioxidants, such as diphenylparapheneylenediamine and ethoxyquin[1], the question arises whether small quatities of alpha-tocopherol must be present in the tissues for their effectiveness, that is, whether they act only as synergists or actually can replace vitamin E. Published results[2-6] on the level of alpha-tocopherol in the organs and tissues of vitamin E-deficient animals contradict themselves to some extent. Thus, for example, Green et al.[2,3] found, after several weeks depletion, 7 µg tocopherol/g in the liver of rabbits while Csallany and Draper[5], with rabbits which were maintained under similar conditions, could find practically no (that is, less than 0.08 µg/g) alpha-tocopherol in the same organ.

For various reasons it seems difficult, with the conventional analytical methods, which are based on the Emmerie-Engel method, to obtain reliable results. The analysis moves to the limit of authenticity at this low concentration of alpha-tocopherol. It also appears questionable whether, at the recovery, the alpha-tocopherol, which is sensitive to oxidation, can be completely protected from decomposition. Therefore, discrepancies could also arise in that the food used to cause depletion contained distinct quantities of residual alpha-tocopherol which, again for the reason of the inadequacy of the analytical methods, could not be determined accurately.

In order to obtain accurate information on the concentration in the tissues during the course of the depletion period, the rats in the first test were given a single oral dose of ^{14}C-labelled alpha-tocopherol acetate and during eight days the radioactivity in the different organs was determined through combustion (Tab.I).

Table I. Alpha-tocopherol content in µg per g of fresh organ weigt in rats after a single dose of 2 mg dl-alpha-tocopherol acetate.

	Organ	½ h	2 h	4 h	24 h	4 Tage①	8 Tage
2	Blut	0,2	1,1	1,9	2,2	0,4	0,3
3	Leber	0,3	8,7	16,7	7,4	1,5	1,3
4	Muskel	0,05	0,2	0,3	0,6	0,4	0,5
5	Niere	0,1	0,5	0,9	2,5	1,1	1,1
6	Nebenniere	0,5	3,1	12,1	62,3	10,6	2,7
7	Herz	0,2	0,6	1,1	1,8	1,5	2,2
8	Hoden	0,05	0,1	0,1	0,9	0,4	0,5
9	Hirn	0,05	0,2	0,2	0,4	0,3	0,6
10	Lunge	1,5	1,5	3,7	7,0	2,0	2,0
11	Milz	0,06	1,7	3,7	11,7	2,7	2,1
12	Haut	0,08	0,4	0,4	1,4	0,7	0,7

Key: 1-days; 2-blood; 3-liver; 4-muscle; 5-kidneys; 6-adrenals; 7-heart; 8-testes; 9-brain; 10-lungs; 11-spleen; 12-skin.

For a longer test period, rats were given ^{14}C-labelled dl-alpha-tocopherol acetate orally on five consecutive days and the radioactivity of the different organs was determined in the same way as in the first test over a time span of 88 days (Tab.II).

Since, as was shown in earlier tests[7], the total radioactivity in the tissues could be associated with alpha-tocopherol and moreover the dl-alpha-tocopherol administered exhibited a considerable specific activity, a sensitive and specific analysis was performed. It could determine concentrations down to 0.05 µg/g.

Table II. Alpha-tocopherol content in µg per g of fresh organ weight in rats after five doses of 1 mg dl-alpha-tocopherol each.

	Organ	4 Tage	11 Tage	18 Tage	32 Tage	47 Tage	68 Tage	88 Tage
2	Blut	4,2	1,6	1,1	0,6	0,2	—	0,1
3	Leber	14,4	6,7	3,1	1,7	0,6	0,7	0,4
4	Muskel	6,4	3,8	2,8	1,6	0,9	0,7	0,2
5	Niere	13,0	6,3	3,8	2,1	0,9	0,7	0,2
6	Nebenniere	97,0	52,5	25,6	11,6	4,1	4,0	2,3
7	Hypophyse	34,2	15,3	11,4	5,8	8,1	3,4	1,5
8	Herz	17,8	10,2	6,3	4,3	1,5	1,4	0,5
9	Hoden	8,4	4,6	3,5	3,3	1,7	2,3	1,0
10	Hirn	5,5	4,0	4,4	4,0	2,9	3,1	3,1
11	Haut	10,5	4,5	3,2	1,1	0,5	1,3	0,4
12	Fett	12,5	14,5	7,0	3,2	1,7	2,3	0,8

Key: 1-days; 2-blood; 3-liver;4-muscle; 5-kidneys; 6-adrenals; 7-hypophysis; 8-heart; 9-testes; 10-brain; 11-skin; 12-fat.

Experimental: The experimental part of this work was performed by Mrs. R. Schiedt.

Animal tests: For test I, male animals weighing 100-150 g were fasted overnight before receiving the single oral dose of ^{14}C-dl-alpha-tocopherol acetate and 4 hours after the administration received normal food. For test II, the animals had an initial weight of 50 g and received a vitamin E-free diet of the following composition:

sugar	50%	swine lard	5%
casein	25%	coconut oil	5%
dehydrated yeast	10%	salt mixture	5%

Per kg of food, 2 g choline chloride and 10 g of the following vitamin mixture were added:

biotin	0.1 mg	p-aminobenzoic acid	70.0 mg
folic acid	0.1 mg	inositol	30.0 mg
thiamin	10.0 mg	ascorbic acid	10.0 mg
riboflavin	10.0 mg	menadiol diphosphate	2.0 mg
pyridoxin	10.0 mg	calcium pentothenate	10.0 mg
nicotinamide	10.0 mg	retinol	3000 IU
sugar	10 g	cholecalciferol	300 IU

^{14}C-dl-alpha-tocopherol acetate was emulsified with Tween 80 (final concentration 2%) and physiological NaCl-solution. The rats of test I received a single dose of 0.5 ml emulsion, corresponding to 2 mg dl-alpha-tocopherol acetate; those of test II received 0,25 ml five times, that is, a total of 5 mg dl-alpha-tocopherol

acetate in 5 doses.

After each different time period, two rats were decapitated and the blood and organs were removed. Each animal was worked on individually. Double determinations were carried out for all organs. The values given in Tables I and II represent the average values of two animals.

Determination of the alpha-tocopherol content of the organs through measurement of radioactivity: The administered ^{14}C-dl-alpha-tocopherol acetate had a specific activity of 3.7 μC/mg.

The activity existing in the freshly removed organs was determined by the method of Kalberer and Rutschmann[8] through combustion under oxygen to CO_2 and H_2O followed by measurement in a Packard Tri-Carb Scintillation Counter (Mod. 314 A).

Results and discussion: The results are summarized in Tables I and II. It results that after administration of 2 mg dl-alpha-tocopherol acetate, the increase of the alpa-tocopherol concentration in the different organs is different. In the liver the maximum is reached after four hours, in the other organs only after 24 hours and in the brain only after eight days, It seems therefore that vitamin E is taken up primarily by the liver and in contrast to vitamin A which is stored there for a long time, is relatively quickly delivered to other organs and tissues. The tests confirm the previously found selective storing capacity for vitamin E in the adrenals and hypophysis. The monitoring of the vitamin E-concentration over a long period of time shows clearly that after a relatively short-term fall there exists a distinct tendency to maintain a certain minimal quantity of vitamin E in the organs. It is noteworthy that 88 days after administration of dl-alpha-tocopherol, values of 0.1 to 3.1 μg/g were found. After an 88-day depletion period, the radioactive alpha-tocopherol present always existed in the magnitude of the quantities found for other vitamins with normal supply. The lowest value, 0.1 μg/g, found after 88 days was in the blood. In conformity therefore, an increased hemolysis-slope was the only symptom determined in these animals.

When one takes into account that so-called vitamin E-free food probably always contains traces of vitamin E - Green et al.[2] have, for example, determined that the food they used contained 2.4 μg/g -and also considers the distinct storing capacity of the organism for alpha-tocopherol, then is it understood that only with great difficulty could a complete vitamin E-depletion be obtained.

Summary

1. The radioactivity in various organs of rats was determined after peroral administration of 2 and 5 mg of ^{14}C-dl-alpha-tocopherol acetate at intervals of half an hour to 88 days.

2. In all the organs examined, radioactive alpha-tocopherol was found in concentrations of 0.1 μg/g (blood) to 3.1 μg/g (brain), even 88 days after administration.

3. The adrenals and hypophysis were found to be especially enriched with alpha-tocopherol.

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HYPERVITAMINEMIA TEST IN NORMAL HUMAN SUBJECTS DIFFERENCE IN ABSORPTION OF α-TOCOPHEROL IN THE FREE STATE AND AS ACETATE ESTER

by H. Gounelle, C. Marnay and M. Blondin

Two of us showed in 1946, even here, that the absorption of a massive dosage of vitamin A caused a rapid rise in the blood axerophtol which reaches its peak after six hours [1]. We asked ourselves whether the same phenomenon would occur concerning another liposoluble vitamin, under the circumstances of vitamin E. We went ahead with α-tocopherol in the free state and tocopherol acetate *.

Method. The dosages were done by the method of Vinet and Meunier [2] as modified [3]. After a taking of blood before breakfast, the subject took 2 grams of vitamin E in the form of either acetate of tocopherol or α-tocopherol in the free state. A second sampling was taken 6 hours after the ingestion when the highest level of vitaminemia occurs. In some subjects additional dosages were made in the 3rd and 24th hour.

key:

1= case number; 2= 0 hour (begin); 3= 6th hour, 4= 24th hour; 5= percentage increase

Numéro observation /	0 heure 2	6e heure 3	24e heure 4	Augmentation en % 5
I BLO	9,36 mg	11 mg	11,1 mg	17,5 %
II LAV	7,2 —	10, —	10 —	38 —
III COL	7,2 —	7,8 —	7,4 —	8 —
IV BAT	4,2 —	5,8 —	4,8 —	38 —
V GE	7,2 —	11,25 —	10,9 —	56 —
VI LAU	7,2 —	12,4 —	—	72 —
VII MEG	6,2 —	11 —	—	77 —
VIII DOR	12,8 —	20 —	—	56 —
IX FOR	10,5 —	11,19 —	—	6 —
X DUC	10,4 —	13,87 —	—	33 —
XI CAR	11,5 —	13,68 —	—	17,5 —
XII GAU	8,1 —	12 —	—	48 —
XIII BRAU	11,4 —	15 —	—	31 —
XIV PAT	7,11 —	8,76 —	—	23,2 —
XV LEM	7,12 —	8,83 —	—	24 —

Table 1 - Hypervitaminemia E test.
Charge of 2 grams of acetate of tocopherol.

1. H. Gounelle, S. Bonfils and Miss Marnay. C. R. de la Soc. de Biol., 1946, vol. 140, p. 980.

* We thank the Hoffmann-Laroche Laboratories which supplied us with the products and funds to permit us to carry out the work.

2. A. Viney and P. Meunier C. R. Acad. Sc., 1941, Vol. 213, page 709.
3. A Guerillot-Vinet and C. Marnay, Techniques de Laboratoire (laboratory techniques), (Loiseleur), Masson et Cie. publishers, Paris, 1953, in the process of being published.

The curve (arrow) of hypervitaminemia E after charge of acetate of tocopherol in 15 normal human subjects shows as an average in the sixth hour an increase of 36.3%.

The method of dosage used gives hhe rate of free tocopherol and thus we sought to reveal the possible presence of acetate of tocopherol in the blood by carrying out a dosage after saponification **. Under these conditions there is no increase in tocopherol in the blood.

key:

1= case number; 2= 0 hour (begin); 3= 6th hour, 4= percentage increase

Numéro observation	0 heure 2	6e heure 3	Augmentation en % 4
I LEC.....	9,6 mg	24 mg	150%
II DUC.....	10,5 mg	17,8 mg	69%
III JAC.....	9,85 mg	20 mg	103%
IV LEC.....	10 mg	21,9 mg	119%
V LAR.....	16,37 mg	35,8 mg	118%
VI DOU.....	10,25 mg	27,4 mg	167%
VII MOL.....	10 mg	16,2 mg	62%
VIII GAU.....	8,3 mg	21,9 mg	163%
IX DOR.....	10,73 mg	24 mg	123%
X BROU.....	10 mg	16,1 mg	61%
XI MOR.....	8,12 mg	13,6 mg	67%

Table II. Hypervitaminemia E test.
Charg e of 2 grams of free α-tocopherol.

In 11 subjects not presenting any clinical problems which could disturb the absorption, a charge of 2 grams of tocopherol in the free state causes a peak in hypervitaminemia E in the sixth hour. The average percentage of rise is 118%. This percentage is therefore three times higher than with acetate of tocopherol.

** The saponification of the esters of the tocopherol in a quantity as small as in the serum poses serious analytical problems.

In order to make this difference in absorption even more precise we studied the hypervitaminemia E test compared in 5 normal human subjects. The time which elapsed between the two "charges" in the same subject was one month.

These five tests show that the vitaminemic absorption in the same subject is approximately three times greater for α-tocopherol in the free state than for the acetate of tocopherol . We find with as much clarity the same percentage of increase as in the preceding two tables.

key:

1= case number; 2= charge of 2 grams of acetate of tocopherol
3= percentage increase; 4= time elapsed between the two charges:4 months;
5= charge of 2 grams of α-tocopherol in the free state
6= percentage increase

N° d'Observation 1	Charge de de 2 g accet. tocophérol 2	Augmentation % 3	Temps écoulé entre les 2 charges : 4 mois 4	Charge de 2 g α tocophérol libre 5	Augmentation % 6
I GAU.....	0 h 8,1 mg 6 h 12 —	48 %		0 h 8,3 mg 6 h 21 —	164 %
II DUC.....	0 h 10, 4 — 6 h 13,87 —	33 —		0 h 10,5 — 6 h 17,8 —	69,5 —
III DOR.....	0 h 12, 8 — 6 h 21 —	56 —		0 h 10,7 — 6 h 24 —	124 —
IV BRO.....	0 h 11,4 — 6 h 15 —	31 —		0 h 10 — 6 h 16,1 —	61 —
V BLO.....	0 h 9,35 — 6 h 11, 1 —	17,5 —		0 h 12 — 6 h 21,17 —	75 —

Table III

CONCLUSIONS

The ingestion of large doses of vitamin E therefore causes an increase in the blood curve of this vitamin occurring in the sixth hour. The increase is different, however, according to whether the product is in the form of tocopherol in the free state or acetate of tocopherol. The absorption of α-tocopherol in the free state causes a rapid rise in the blood curve approximately three times greater than when acetate of tocopherol is taken **.

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METABOLIC EFFECTS OF ALPHA-TOCOPHERYL ACETATE

I. INFLUENCE OF ALPHA-TOCOPHERYL ACETATE ON SOME LIPIDS AND NITROGEN COMPOUNDS OF PLASMA IN HUMAN SUBJECTS[1]

DORIS E. GRAY AND SHIU-MAY LOH

Abstract

Significant increases in phospholipids, total cholesterol, and alpha-2 globulins and significant decreases in amino acids were found in the plasma of healthy medical students following daily ingestion of 100 mg. alpha-tocopheryl acetate (αTAc). The significance of these findings is discussed.

Introduction

Although numerous reports have been published on changes in the lipid and protein constituents of body tissues taken from vitamin E deficient animals, comparatively little information exists on the effect of administration of the vitamin to normal human subjects. Therefore it was decided to ascertain its effect, if any, on some lipids and nitrogen compounds in the plasma of healthy human subjects.

Experimental Procedure

Twelve healthy medical student volunteers were required to fast for 12 hours before each sample of blood was taken. During a preliminary control period four samples of blood were drawn from each subject, at intervals of 3 days, following which 100 mg. alpha-tocopheryl acetate (αTAc) was administered orally each morning for 10 days. Following this 10 day period, four samples of blood were obtained, after 12-hour fasts, and at approximately 3-day intervals. During this time blood levels of alpha-tocopherol were maintained by continuing the daily administration of 100 mg. αTAc.

Free and total cholesterol concentrations of plasma were estimated by the method of Schoenheimer and Sperry (1) and Sperry and Bland (2). Plasma phospholipid phosphorus was determined according to the method of Youngsbury and Youngsbury (3), and Fiske and Subbarow (4), and fatty acids by the method of Stoddard and Drury (5). Amino acid nitrogen was determined using the method of Frame, Russell, and Wilhelmi (6), and electrophoretic separation of the plasma proteins was carried out according to the method described by Liang (7). Where possible all estimations were performed in duplicate.

Results

Table I shows the plasma concentration of total cholesterol, free cholesterol, fatty acids, and phospholipids, in normal subjects, before and after ingestion of 100 mg. α TAc per day. Significant increases in the plasma levels of total and of free cholesterol and of phospholipid phosphorus followed the ingestion of αTAc. Fatty acid levels were not significantly influenced by the αTAc.

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Contribution from the Department of Physiology, University of Hong Kong, Hong Kong.

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TABLE I

CONCENTRATION OF SOME LIPIDS IN HUMAN PLASMA, BEFORE AND FOLLOWING INGESTION OF ALPHA-TOCOPHERYL ACETATE

Plasma lipids	N	Control period Mean ± S.E.M.	Test period Mean ± S.E.M.	*P* value
Free cholesterol	12	54.9 ± 3.26	60.2 ± 3.15	<0.01>0.001
Total cholesterol	12	166.0 ± 9.64	178.8 ± 10.28	<0.01>0.001
Phospholipids	12	180.8 ± 10.96	204.4 ± 11.34	<0.01>0.001
Fatty acids	12	233.1 ± 12.18	245.2 ± 7.51	

NOTE: N is the number of values, each itself the mean from four samples. Estimations are expressed as mg. per 100 ml. plasma.

TABLE II

CONCENTRATION OF TOTAL AMINO ACIDS, IN HUMAN PLASMA, BEFORE AND FOLLOWING INGESTION OF ALPHA-TOCOPHERYL ACETATE

	N	Mean	±S.E.M.
Control period	6	6.6	±0.05
Test period	6	6.1	±0.19
P value	<0.05	>0.02	

NOTE: N is the number of values, each itself the mean from four samples. Estimations are expressed as mg. per 100 ml. plasma.

TABLE III

DISTRIBUTION OF PLASMA PROTEIN BY ELECTROPHORESIS

Component	N	Control ±S.E.M.	N	Test ±S.E.M.	*P* value
Albumin	24	53.7±0.98	24	52.8±1.49	
α-1-Globulin	24	4.1±0.27	24	4.1±0.45	
α-2-Globulin	24	8.3±0.31	24	9.3±0.45	<0.001
β-Globulin	24	12.6±0.53	24	12.5±0.45	
δ-Globulin	24	9.8±0.53	24	9.2±0.49	
γ-Globulin	24	11.5±1.02	24	12.1±1.39	

NOTE: Values are expressed as per cent total protein. N is the number of samples.

Table II shows the plasma concentration of amino acid nitrogen in the same subjects, before and after the ingestion of 100 mg. αTAc per day for 10 days. The amino acid nitrogen was significantly lowered following αTAc administration. Table III shows the electrophoretic distribution of plasma proteins before and after administration of the vitamin. A significant increase occurs only in the alpha-2 globulin fraction.

Discussion

Significant increases in total cholesterol, free cholesterol, and phospholipids in the plasma of healthy subjects were found after a daily intake of 100 mg. αTAc for 10 days. These increases in plasma lipids indicate clearly that the vitamin is implicated in the metabolism of fats. Similarly, a significant increase in the alpha-2 globulin component of plasma proteins, and a significant *decrease* in the plasma amino acids indicate that the vitamin is in some way connected with the metabolism of protein.

Increases in plasma cholesterol levels following vitamin E administration have been reported in both atherosclerotic rabbits (8) and atherosclerotic patients (9). Hove and Hove (10) observed that lipid depletion of arachodonate from the livers of rats fed with tri-*o*-cresyl phosphate can be prevented by administration of vitamin E, and further evidence that the liver is involved in the metabolism of vitamin E has been presented by Quaife (11), who demonstrated that the tissue most influenced by feeding alpha-tocopherol is liver.

The work of many investigators has suggested that a link may exist between vitamin E and protein metabolism. Dam (12) found that superimposing a protein deficiency on a vitamin E deficiency reduced the survival time in rats in comparison with vitamin E supplemented controls; Hove and Harris (13) demonstrated that the protein-efficiency ratio (total gain in body weight/protein consumed) was increased by the addition of alpha-tocopherol to a diet deficient in that vitamin; others have observed marked changes in certain tissues of experimental animals maintained on a diet deficient in vitamin E. Thus Olson (14) has shown that hepatic necrosis, which develops when weanling rats are fed diets devoid of vitamin E and low in sulphur amino acids, is prevented by administration of alpha-tocopherol and methionine, and that coenzyme A (CoA) content of liver varies inversely with the degree of hepatic necrosis. He has also shown (15) that acetyl CoA, a thioester of acetate and CoA, which is the active two-carbon fragment through which carbon and hydrogen of all oxidizable substrates of fat, carbohydrate, and protein must pass, can be increased by administration of vitamin E. That these changes involve sulphur metabolism has been demonstrated by Yang and Olson (16), who report that normal rats fed S^{35}-labelled cystine incorporate the cystine sulphur in CoA.

Milman, Silides, and Milhorat (17) found that less amino acid was released, *in vitro*, from the skeletal muscle of vitamin E deficient hamsters than from that of paired normal controls, and they have suggested that the reduction in protein breakdown may be secondary to a decrease in protein synthesis. In our laboratory, vitamin E administration was found to be attended by significantly lower concentrations of amino acid in plasma and by increased plasma protein concentration, suggesting that the increase in protein is made at the expense of the amino acids.

The electrophoretic patterns of plasma proteins show a significant increase in the alpha-2 globulin component following the ingestion of αTAc. Lewis,

Quaife, and Page (18) have reported that alpha-tocopherol is carried mainly in the alpha-2 globulin fraction of plasma proteins as lipoprotein complexes. Thus it seems likely that this fraction of protein is transported in plasma by the alpha-tocopherol as a lipoprotein complex. Olson (14) suggests that vitamin E protects the supply of sulphur amino acids in the body when they are scarce; Latner (19) has made similar statements based on clinical studies on patients with hepatic disorders. Olson and Dinning (20) have shown that hepatic CoA levels are lowest in necrosis, somewhat higher in animals on deficient diets but showing a little hepatic necrosis, and highest of all in those animals maintained on diets with alpha-tocopherol supplement. Thus one might expect an increase in synthesis of any compound which is dependent for its synthesis upon CoA, and it is suggested that, irrespective of substrate, the presence of alpha-tocopherol stimulates the synthetic process wherever CoA is required. According to Oliveira (21), vitamin E may be regarded as a component of a biological system and not merely as an antioxidant. Indeed it is generally acknowledged now that vitamin E has a specific and primary function, and a secondary non-specific action as a physiological antioxidant.

Nason and Lehman (22) have demonstrated in particulate preparations of rat skeletal muscle, the presence of a lipid cofactor functioning between cytochrome-*b* and cytochrome-*c* in the electron transport system, which can be substituted for specifically by the tocopherols, although in itself it contains no free tocopherol. These workers have made the tentative suggestion that the "co-factor or tocopherol acts as cementing material for maintaining cytochromes-*b* and *c* in correct spatial relation for optimal electron transfer". Thus the addition of vitamin E might result in the production of more energy, which would then be available for synthetic reactions. Whether vitamin E stimulates the synthesis of ATP directly, which in turn (provided the constituent molecules are available in sufficient quantity) could provide the energy for increased synthesis of CoA, remains to be established in the normal animal. Olson (15) has shown, however, that the amount of CoA in necrotic liver is inversely proportional to the degree of necrosis, and this would argue a dependence on vitamin E for CoA synthesis. It is, therefore, an interesting possibility that the action of vitamin E in increasing the concentration of lipids and protein of plasma is general, rather than a specific effect, dependent on the supply of CoA, which in turn could be dependent on the supply of ATP. Chernick and Schwarz (23) have concluded from their experiments with liver tissue from vitamin E deficient animals that the 'respiratory lesion' may involve a defect in ATP synthesis. In our laboratory, preliminary experiments with rat liver homogenates from animals maintained on large amounts of αTAc as a dietary supplement yield results which suggest that the liver cell from these animals is more active in the synthesis of fats and protein than is the cell of the normal liver. We are presently engaged in attempting to measure small amounts of ATP, as well as CoA in the liver cell, under varying conditions involving sulphur and vitamin E relationship.

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METABOLIC EFFECTS OF α-TOCOPHERYL ACETATE
II. INFLUENCE OF α-TOCOPHERYL ACETATE ON CHOLESTEROL AND PHOSPHOLIPID SYNTHESIS IN RAT LIVER HOMOGENATES

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Gray and Loh (1) have shown previously that dietary α-tocopheryl acetate (α TAc) brings about increases in phospholipids, total and free cholesterol in the plasma of healthy human subjects. It seemed probable that the dietary αTAc affected the liver cell itself, the blood being merely a transport medium. This hypothesis was investigated, using rat liver homogenates from animals maintained for 8 days on a standard diet supplemented with 100 mg αTAc per day per rat.

EXPERIMENTAL

Male albino rats of 100 to 150 g weight were divided at random into two groups. Both groups, test and control, were maintained on a standard diet consisting of rice, bran, and meat mash, for 8 days, the test group receiving in addition a daily dietary supplement of 100 mg αTAc per rat. The animals were then fasted overnight, decapitated, and the livers removed and homogenized by an MSE homogenizer, in a bicarbonate buffer, described by Sinex, McMullen and Hastings (2). 1 ml homogenate (20 per cent) from each liver was pipetted into a series of flasks containing 22 μM glycerol as substrate, and 10 μM ATP. Total volume was 4 ml. At the same time, 1 ml homogenate was pipetted into a flask which contained 3 ml buffer only. Incubation was carried out in a Warburg apparatus at 37° for various time periods.

Following incubation, the samples were analyzed for their content of phospholipid, total and free cholesterol Phospholipid phosphorus was determined by the method of Youngsburg and Youngsburg (3) and Fiske and SubbaRow (4); total and free cholesterol by the method of Schoenheimer and Sperry (5). Ester cholesterol was calculated by difference. The results were analyzed using the "Analysis of Variance" technique of Snedecor (6).

RESULTS

Inspection of Table I indicates that dietary αTAc affected the resting levels of phospholipids, total and ester cholesterol, as well as the synthesis of these

TABLE I

Mean Lipid Concentration in Respiring Rat Liver Homogenates in the Presence of Added Substrate and Cofactor.

Lipids determined	Experimental conditions	Incubation time (*min*) 0	30	90	180	N[a]	P value
Phospholipids	Test	2.826	3.073	3.159	3.301	14	
	Control	2.660	2.813	2.880	3.017	14	
	Difference	0.166	0.260	0.279	0.284		<0.01
Total cholesterol	Test	0.504	0.545	0.537	0.606	9	
	Control	0.458	0.470	0.515	0.526	9	
	Difference	0.046	0.075	0.022	0.080		<0.02>0.01
Ester cholesterol	Test	0.296	0.322	0.305	0.357	9	
	Control	0.250	0.239	0.285	0.279	9	
	Difference	0.046	0.083	0.020	0.078		0.001
Free cholesterol	Test	0.208	0.224	0.232	0.249	9	
	Control	0.207	0.231	0.229	0.247	9	
	Difference	0.001	-0.007	0.003	0.002		

[a] The number of animals.
Substrate: 22 μM glycerol.
Cofactor : 10 μM ATP.
Values are expressed as mg per 100 mg tissue.
Test homogenates are prepared from livers of animals maintained on a basel diet, with α TAC added as supplement.

lipids by rat liver homogenates during subsequent incubation with glycerol and ATP present in the medium. Free cholesterol did not differ significantly from the control values. In Table II it is seen that αTAc has a similar effect

TABLE II

Mean Lipid Concentration in Respiring Rat Liver Homogenates in the Absence of Added Substrate and Cofactor.

Lipids determined	Test	N[a]	Control	N[a]	Difference	P value
Phospholipids	2.960	14	2.754	14	0.206	<.05
Total cholesterol	0.527	9	0.468	9	0.059	.05
Ester cholesterol	0.304	9	0.226	9	0.078	.02
Free cholesterol	0.223	9	0.216	9	0.007	

[a] The number of animals.
Values are expressed as mg per 100 mg tissue.
Test homogenates are prepared from livers of animals maintained on a basal diet, with α TAc added as supplement.
Incubation time: 180 min.

in the absence of substrate. However, the formation of these lipids in general proceeds at a faster rate when substrate and ATP are present in both the

test and control samples.

DISCUSSION

The results of these experiments lend support to the hypothesis, suggested by Gray and Loh (1) that αTAc has a site of action in the liver with respect to the metabolism of lipids. That the liver is at least one "target" for the action of vitamin E is not a new idea, for it has been demonstrated by many investigators that in the experimental animal, the hepatic necrosis following a dietary regimen deficient in vitamin E can be prevented by administration of the vitamin. Rosecan, Rodnan, Chernick and Schwarz (7) found that vitamin E administration to rats prevented the hepatic necrosis and the "respiratory lesion" induced by a Torula yeast diet deficient in the vitamin; Chernick and Schwarz (8) and McCay and Caputto (9) have published data from which they conclude that the cause of this biochemical lesion in necrotic liver slices from vitamin E deficient rats lies in the oxidative phosphorylation system. Chernick and Schwarz (8) suggest that a defect in ATP synthesis may be involved. Chernick, Moe, Rodnan and Schwarz (10) have reported that rat liver homogenates prepared from animals with hepatic necrosis failed to respire at normal rates with every substrate tested, including glucose, fumarate, succinate and ketoglutarate. Schwarz (11) reported the prevention of necrotic liver degeneration in young rats by the administration of vitamin E, and suggested that the "primary metabolic defect is closely related to the citric acid cycle". Thus there is general agreement that vitamin E plays a metabolic role in the aerobic phase. It has been demonstrated, by Nason and Lehman (12) in particulate preparations of rat skeletal muscle, that the vitamin has one site of action in the electron transport system, at the cytochrome c level, and therefore we may suspect that in the liver the vitamin behaves in a similar, if not identical, chemical system. Simon, Gross and Milhorat (13) have reported that following intravenous administration of C^{14}-labeled tocopherol, 60 to 80 per cent of the radioactivity is found in the mitochondrial and microsomal fractions of mice liver homogenates, the isotope concentration in the mitochondria being approximately twice that in the microsomes. Since aerobic enzymes are present in the mitochondria of the cell, one might expect vitamin E to be there also, if it is intimately concerned with respiration and ATP synthesis. Slater and his co-workers (14–17) have proposed a scheme by which the vitamin functions as an integral part of the respiratory chain.

Olson (18) and Olson and Dinning (19) have demonstrated that changes occur in coenzyme A (CoA) content of necrotic rat liver, and that quantitatively these changes correlate inversely with the degree of necrosis. Olson's suggestion that an "etiological relationship of reduced synthesis of CoA to hepatic necrosis, involving vitamin E and sulphur amino acid metabolism is likely", would argue a dependence on vitamin E for CoA synthesis. That these changes involve sulphur metabolism has been demonstrated by Yang and Olson (20) using S^{35}-labeled cystine.

In the present experiments significant increases were found in the resting

levels of liver phospholipids, and in total and ester cholesterol, from animals maintained on a standard diet supplemented with αTAc, when compared with controls. That the liver cell itself is in some way altered is demonstrated by its subsequent metabolic behaviour in the presence of added substrate. In all instances the respiring homogenates, from αTAc-fed animals, synthesized more phospholipid, total and ester cholesterol during a 3-hour incubation period, than did the control animals, whether substrate was present or not.

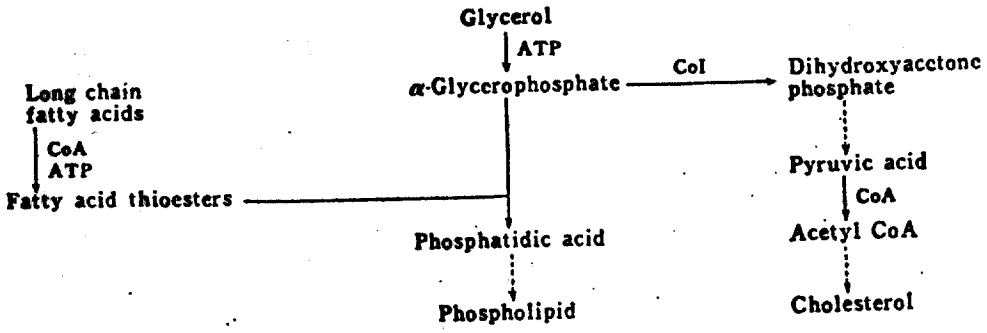

FIG. 1 *Metabolic Pathways in the Synthesis of Cholesterol and of Phospholipid.*

Fig. 1 indicates the metabolic pathways for the synthesis of cholesterol and phospholipid, and the relative position of CoA and ATP in the scheme. Whether the increases in formation of lipids involve primarily increases in ATP synthesis, and secondarily increases in CoA synthesis, has not yet been established. If vitamin E increases the synthesis of ATP directly, which in turn could provide energy for increased synthesis of CoA, the findings of several investigators could be brought into logical perspective. In this laboratory we are currently determining the influence of dietary αTAc on the ATP and CoA content of rat liver, and preliminary experiments strongly suggest that the liver of vitamin E-fed animals contains more CoA per unit of deoxyribonucleic acid than do control livers (21).

It is recognized that the amount of lipids determined in these studies are the resultants of the two processes of synthesis and of degradation. *De novo* synthesis cannot be demonstrated by the methods used, and hence an alternative explanation of the apparently increased synthesis is possible. If the degradation process is inhibited by the presence of tocopherol, an apparent increase in synthesis would occur. Nevertheless, in the light of the evidence that the vitamin stimulates the transfer of electrons at the cytochrome c level (12), it is unlikely that tocopherol acts in the present experiments to inhibit degradation processes. From this laboratory, the author (22) has reported that guinea pig diaphragm forms significantly greater amounts of pyruvic acid, when the tissue is incubated with added α-tocopherol in the form of tocopherol-albumin conjugates, in the presence of substrates, alanine, glucose, and glutamic acid. This provides further confirmatory evidence that the effect of tocopherol is general and not specific.

SUMMARY

1. Male albino rats maintained for 8 days on a standard diet, with 100 mg αTAc per rat added as daily dietary supplement, had significantly higher levels of liver phospholipids, total and ester cholesterol than their controls.

2. During the course of incubation, with glycerol as substrate, the test homogenates synthesized significantly greater amounts of these lipids than the control homogenates.

3. The significance of these findings is discussed.

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THE JOURNAL OF VITAMINOLOGY 6, 62–67 (1960)

METABOLIC EFFECTS OF α-TOCOPHERYL ACETATE
III. INFLUENCE OF α-TOCOPHERYL ACETATE ON COENZYME A, ADENOSINE TRIPHOSPHATE AND ADENOSINE DIPHOSPHATE IN RAT LIVER

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It has been demonstrated by Gray (1) that dietary α-tocopheryl acetate (α-TAc) increases the resting levels, and the synthesis *in vitro*, of phospholipids and of cholesteryl esters, by rat liver homogenates, with glycerol as substrate and adenosine triphosphate (ATP) as added cofactor. Since coenzyme A (CoA) and ATP are metabolic requirements for the synthesis of these compounds (2–5), the present investigation was undertaken to determine if the increase in formation of phospholipids and cholesteryl esters might be explicable in terms of increasing ATP and CoA of liver, taken from rats fed dietary α-TAc as additions to a stock diet.

EXPERIMENTAL

General — Male albino rats of 100 to 150 g weight were divided at random into two groups. Both groups were maintained on a diet consisting of rice, bran and meat mash, for 20 days, one group receiving in addition a daily dietary supplement of 100 mg α-TAc per rat. The animals were killed by decapitation under chloroform anaesthesia, and the livers removed and weighed.

Preparation of Tissue Extract for ATP and ADP Estimation — 2.5 g liver was minced, homogenized at once in 2 volumes of ice-cold 0.1 *M* tris (hydroxymethyl)-aminomethane buffer at pH 7.5, and 1 ml of 18% (v/v) perchloric acid was added to precipitate the protein. After centrifugation the precipitate was washed twice with 2 ml buffer, and washings and supernatant solutions were combined. All manipulations were carried out at 0°. The pH of the extracts was adjusted to 7.5 with KOH, and the volume of the extracts brought up to 25 ml. Storage at 5° for several hours precipitated the perchlorate as the potassium salt. Following centrifugation the precipitate was removed, and the supernatant used for determination of ATP and adenosine diphosphate (ADP).

Preparation of Tissue Extract for CoA Assay — The remainder of the

liver was minced and homogenized in boiling water, placed in a boiling water bath for 5 minutes, rehomogenized and made up to a volume of 50 ml, from which aliquots were taken. Following centrifugation, the supernatant was used for CoA assay, and the precipitate used for the determination of the deoxyribonucleic acid (DNA) content.

Determination of ATP — The method applied was a modification of that of Holloway (6), in which the 1,3-diphosphoglyceric acid formed from 3-phosphoglyceric acid and ATP is converted to 3-phosphoglyceraldehyde with the concomitant oxidation of reduced diphosphopyridine nucleotide (DPNH). The decrease in absorption at 366 mμ is therefore proportional to the amount of ATP present. To a cuvette is added 3 ml sample, 0.4 ml of a substrate mixture (4×10^{-3} *M* $MgSO_4$; 3×10^{-3} *M* cysteine; 6×10^{-3} *M* 3-phosphoglyceric acid), and 0.04 ml 1.2×10^{-2} *M* DPNH. The extinction at 366 mμ was then measured, and the reaction started by addition of 0.04 ml enzyme mixture (4 mg per ml phosphoglyceraldehyde dehydrogenase; 1 mg per ml phosphoglycerokinase). The drop in extinction in 10 minutes was determined. This decrease is proportional to the concentration of ATP.

Determination of ADP The enzyme system used was that of Kornberg and Pricer (2) as follows:

(1) ADP + phospho-enol-pyruvate ⇌ ATP + pyruvic acid

(2) Pyruvic acid + DPNH + H^+ ⇌ lactic acid + DPN^+

The assay system was as follows: 3 ml sample, 0.15 ml substrate mixture (10^{-2} *M* phospho-enol-pyruvate; 1.3 *M* KCl, 0.04 *M* $MgSO_4$), 0.10 ml 5×10^{-3} *M* DPNH, 0.02 ml 1 mg per ml lactic dehydrogenase. The contents of the cuvette were then mixed, the extinction at 366 mμ determined, and the reaction started by addition of 0.02 ml 1 mg per ml pyruvate kinase. The drop in extinction in 10 minutes was determined. This decrease is proportional to the concentration of ADP.

Determination of CoA — An aliquot of the supernatant was subjected to the procedure of Novelli and Schmetz (7), by which CoA is hydrolyzed, releasing pantothenic acid. Pantothenic acid is then assayed by a microbiological method (8) using *Lactobacillus arabinosus* 17—5 (ATCC 8014) grown in Difco "Pantothenate medium". Bacterial growth was determined turbidimetrically at 690 mμ after incubation for 18 hours at 33°.

Determination of DNA DNA was determined by the method of Ceriotti (9).

Analysis of Results The data were analysed using the "Analysis of Variance" technique of Snedecor (10).

RESULT

Inspection of Table I reveals that dietary α-TAc brings about a significant increase in ATP, ADP and CoA of rat liver. It was observed that although the tocopherol-fed animals had significantly larger livers than the control animals, the liver DNA content of both groups remained relatively constant (Table II). The concentrations of CoA, ADP and ATP were therefore ex-

TABLE I

Influence of Dietary α-Tocopheryl Acetate on Mean Levels of CoA, ATP and ADP in Rat Liver

N, number of animals. Values are expressed as μmoles per mg DNA.

Compound determined	Test	N	Control	N	Test control	P value
CoA	0.130	10	0.077	10	0.053	0.001
ATP	0.230	10	0.140	10	0.090	0.01
ADP	0.260	10	0.190	10	0.050	0.05

TABLE II

Influence of Dietary α-Tocopheryl Acetate on Mean Weight of Rat Liver, and Its Mean DNA Content

N, number of animals. Liver weights are expressed in g per liver. Liver DNA is expressed in mg per liver.

	Test	N	Control	N	Test control	P value
Liver weight	6.54 ± 0.37[a]	10	5.63 ± 0.219	10	0.91	< 0.02
Liver DNA	10.69 ± 1.54	10	10.05 ± 1.31	10	0.64	0.4

[a] S. E. M.

pressed with reference to the liver DNA.

DISCUSSION

That CoA and ATP are both metabolic requirements for the synthesis of phospholipid and cholesterol has been well-established. Thus, in our laboratory, when dietary α-TAc was found to bring about an increased synthesis of these lipids in liver tissue (1), the suggestion arose that such increase might be attributable to increased formation of CoA and ATP. In the experiments reported in this paper, the striking increase in CoA and ATP of rat liver from animals fed a diet supplemented by α-TAc confirms this hypothesis. Previous findings from this laboratory (11, 12) had led to the suggestion that α-tocopherol acts in a chemical system required for the synthesis of a variety of compounds. Such syntheses could be explicable in terms of increased ATP and CoA levels as a result of adding vitamin E *in vivo* and *in vitro*.

Although it is generally agreed that vitamin E exerts its influence primarily in the respiratory chain, the evidence for this is usually deduced. Direct evidence of the participation of vitamin E in a specific chemical system has been conspicuously absent.

Donaldson, Nason, Moore and Garrett (13) in particulate preparations of rat skeletal muscle, have demonstrated that extraction by the non-polar solvent, isooctane, inactivates the DPNH-cytochrome c-reductase and DPNH oxi

dase systems which can be restored specifically by α-tocopherol, provided the particles are aged. Bouman and Slater (14) and Deul, Slater and Veldstra (15) have isolated significant amounts of α-tocopherol from pig heart mitochondrial particles and were able to restore the activity of cytochrome c-reductase in their isooctane-extracted particles by the addition of α-tocopherol. In addition, Marinetti, Erbland, Morrison, and Stotz (16) and Morrison, Crawford and Stotz (17) have isolated a lipid fraction from pig heart which restores the cytochrome c-reductase activity of isooctane-extracted particles. This fraction contains keto esters. Festenstein, Heaton, Lowe and Morton (18) isolated a compound from animal tissues having an absorption maximum of 275 mμ which they referred to as '*SA*', and later, on finding it to be a quinone, termed it *ubiquinone* (19). Subsequently Crane, Hatefi, Lester and Widmer (20) isolated a quinone from electron transport particles (ETP) of beef heart with an absorption peak of 275 mμ which they first termed Q_{275}, later called *mitoquinone* (21), and finally, on the recognition that this compound functions as a coenzyme, termed it *coenzyme Q* (CoQ). This compound has multiple forms and is not identical with any of the known fat-soluble vitamins (22). The multiple forms differ from one another in the length of the isoprenoid side-chain, as well as the substituents of the ring system.

No evidence that CoQ is a component of the DPNH chain was produced, its activity being confined to the oxidation of succinate in isooctane extracted ETP (22). From this extensive work it appears that CoQ is not the factor which is derived from α-tocopherol, and which is responsible in our experiments for the increased formation of CoA, ATP and ADP.

Simon, Eisengart, Sundheim and Milhorat (23) have isolated 2-(3-hydroxy-3-methyl-5-carboxy pentyl)-3,5,6-trimethyl benzoquinone and its γ-lactone from human urine following large doses of vitamin E. These isolated compounds are metabolic derivatives of α-tocopherol. The authors have published a hypothetical scheme which accounts for the production of this breakdown product, involving the oxidation of the terminal methyl group to a carboxyl group, and the subsequent esterification with CoA. Our hypothetical scheme incorporates this idea (Equation 2).

Recently, Harrison (24) and Clark, Kirby and Todd (25) have proposed a scheme in which quinoid compounds function as electron carriers, reacting with inorganic phosphate to form high energy phosphate compounds.

McMurray and Lardy (26) have made the interesting observation that phosphorylation by isolated submitochondrial particles of rat liver is stimulated by addition of reduced CoA in low concentrations. Thus they suggest the possibility that CoA may play a role as cofactor in oxidative phosphorylation. In order to incorporate these isolated pieces of information into a coherent chemical explanation we have proposed the following scheme:

(1) $AH_2 + B + CoA \rightleftharpoons A \sim SCoA + BH_2$
(2) $A \sim SCoA + \alpha\text{-}T \rightleftharpoons A + CoAS \sim \alpha\text{-}T$
(3) $CoAS \sim \alpha\text{-}T + Pi \rightleftharpoons CoAS \sim P + \alpha\text{-}T$
(4) $CoAS \sim P + ADP \rightleftharpoons CoA + ATP$
(5) Dephospho-CoA + ATP $\rightleftharpoons$ CoA + ADP

In this scheme an increase in CoA would result in increases in ATP, which would in turn bring about an increase in ADP. Addition of α-tocopherol would first of all stimulate synthesis of ATP. This is then used for the synthesis of CoA and CoA can in turn stimulate the formation of ATP. It is interesting that the molar ratio of ATP : CoA in our experiments is approximately 2, which is reasonable in view of the high molecular requirement of phosphate for CoA synthesis.

We are currently attempting the isolation of the derivative responsible for bringing about increased formation of ATP, ADP and CoA.

SUMMARY

1. Male albino rats fed a stock diet supplemented daily with 100 mg α-tocopheryl acetate per rat had significantly higher concentrations of CoA, ATP and ADP than did their controls.
2. The significance of these findings is discussed.

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METABOLIC EFFECTS OF α-TOCOPHERYL ACETATE
IV. DEOXYRIBONUCLEIC ACID OF THE RAT LIVER CELL, FOLLOWING DAILY DIETARY SUPPLEMENTS OF α-TOCOPHERYL ACETATE

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It is well-established that amount of deoxyribonucleic acid (DNA) per somatic cell for a given species remains constant, irrespective of the nutritional status of the animal (1). Therefore, any variation in the mean amount of DNA per organ must be attributed to variations in either the mass or the number of cells of the tissue concerned. In the course of an investigation into the activity of certain enzymes of rat liver following a period of daily administration of α-tocopheryl acetate (αTAc) it was observed that the DNA content per unit weight of liver was significantly increased. This paper deals with the findings in respect of DNA, and the calculation from these data, of liver cell number and cell mass. Paper V, which follows the present paper, is concerned with the enzyme activities, and other metabolic determinations, from the same experimental material.

EXPERIMENTAL

Male weaning rats were divided into three groups and maintained for periods of four to six weeks on a vitamin E-free, low fat diet of the following composition, by weight: carbohydrate 67%, fat 3%, and protein 27%. Two of the groups were given daily supplements of vitamin E in the form of αTAc as follows: 0.5 mg (control group), 100 mg (E-excess group). The third group (E-deficient group) received no αTAc. After the animals had been killed by decapitation, the livers were removed and weighed. An aliquot of the liver was homogenized and extracted, using the method of Ogur and Rosen (2), and DNA was determined according to the procedure of Burton (3).

RESULTS AND DISCUSSION

Table I shows the effect of αTAc, and of deprivation of tocopherol in the young rat. It is seen that the E-deficient animals fail to grow normally, compared with the control and E-excess groups. The striking weight loss of the E-deficient animals is obviously due to loss of appetite, for they practically ceased eating, apparently because of the unpalatability of the very low fat

diet. On the other hand, when αTAc was added as a daily supplement, whether in normal or excess amounts, appetites were restored and they grew at a substantially normal rate.

The liver weights, to a considerable extent, paralleled general growth rates, and it is seen that the ratio of body weight to liver weight is essentially constant, irrespective of whether the animal is deprived of vitamin E or not.

No influence of vitamin E deficiency was seen in the DNA content per unit weight of liver; however, the administration of αTAc in excess amounts significantly increased the DNA content when compared with the control or vitamin E-deficient animals. Assuming a constant amount of DNA per mean cell nucleus, the average cell number can be calculated. Similarly the cell mass in grams, which is 1/cell number, can be computed. From these calculations (Table I), it is evident that the number of cells per gram of liver tissue is significantly greater in rats fed an excess of αTAc. As a corollary, the cell mass is significantly smaller.

Several workers have studied the relationship between vitamin E and the nucleic acids, using the classical approach of inducing a characteristic deficiency in the experimental animal, and deducing vitamin function by observing the changes in comparison with non-deficient animals. Thus Dinning, Sime and Day (4) found, in the rabbit, that vitamin E deficiency fails to affect the incorporation of C^{14}-labeled formate into DNA of liver tissue, although there is extensive incorporation of C^{14}-labeled guanylic acid (5); P^{32} is incorporated into nucleic acids of all tissues studied, including liver, at considerably increased rates in E deficiency (6). In E-deficient rats (7) the incorporation of C^{14}-labeled formate into nucleic acids is twice that of normal controls. These findings were interpreted as an indication that "regulation of the turnover rate of nucleic acids is believed to be a primary metabolic function of vitamin E". In spite of the increased turnover rate in E deficiency, the concentration of nucleic acids remains unchanged (8). Other workers have found that the daily administration of small amounts of αTAc to normal rats fails to modify the amount of DNA in liver, but if the supplement is increased to 100 mg per day a significant increase in the DNA occurs (9). Niesar (10) found that the infusion of tocopherol in Tween 60 into a neck vein of normal rats leads to significant increases in DNA of liver as well as in other tissues.

Our findings agree with the general body of scientific opinion on this matter. Vitamin E deficiency does not alter the concentration of DNA, per unit wet weight of rat liver, despite drastic changes in body and liver weights, but the administration of massive doses of αTAc results in significantly higher levels of liver DNA in the rat. It is recognized that polyploidy in liver complicates any attempt to assign a constant amount of DNA per cell. Therefore any value obtained from organ analysis is necessarily an average value. Nevertheless, other workers who have studied this phenomenon in some detail have concluded that a mean cell content of DNA is the most stable and reliable parameter of the cell (1). The increased amounts of DNA found

liver following administration of αTAc can be referred to the cellularity of the tissue, assuming a mean cell amount of 9.3×10^{-12} g from which the cell mass can be calculated (Table I). It is apparent, therefore, that αTAc in large doses, in the rat, stimulates hyperplasia in the liver, and it follows that the cell mass is correspondingly reduced.

TABLE I

Influence of Dietary α-Tocopheryl Acetate on Mean Body Weight, Liver Weight, Deoxyribonucleic Acid, Cellularity and Mass of Rat Liver

	Control (N=6)	E-deficient (N=6)	E-excess (N=6)
Body weight (g)	105.6 ± 4.6	53.5 ± 4.4	105.6 ± 3.5
		$P \ll 0.001$	$P \ll 0.001$
Liver weight (g)	4.77 ± 0.43	2.58 ± 0.10	5.27 ± 0.34
		$P < 0.001$	$P \ll 0.001$
Ratio $\frac{\text{Body weight}}{\text{Liver weight}}$	2.2	2.1	2.0
		$P \ll 0.001$	
DNA per liver (mg)	4.88 ± 0.37	2.87 ± 0.23	7.17 ± 0.52
		$P < 0.001$	$P \ll 0.001$
			$P < 0.01$
DNA per g (mg)	1.03 ± 0.07	1.11 ± 0.06	1.37 ± 0.05
		$P < 0.01$	
		$P < 0.01$	
Cellularity per liver ($\times 10^6$)	524 ± 41	309 ± 24	771 ± 55
		$P < 0.001$	$P \ll 0.001$
			$P \ll 0.01$
Cellularity per g ($\times 10^6$)	110 ± 7.5	120 ± 6.5	146 ± 5.3
		$P < 0.01$	
			$P < 0.01$
Cell mass ($\times 10^{-3}$ g)	9.1 ± 0.58	8.6 ± 0.35	6.6 ± 0.48
		$P < 0.01$	

Control rats were fed 0.5 mg α-TAc per rat per day.
E excess rats were fed 100 mg α-TAc per rat per day.
N is the number of animals.

In previous experiments, rats maintained on a diet of rice, bran and meat mash, when fed large amounts of αTAc (11) did not show any effect of the vitamin on DNA concentration in liver. However, these animals were not weaning rats, and it has been amply demonstrated that the most pronounced effects of vitamin E deprivation or administration are to be seen in young animals.

SUMMARY

1. Male weaning rats were divided into three groups and maintained for several weeks on a low fat, vitamin E-free diet.
2. Two of the groups termed control and E-excess groups were given, respectively, daily supplements of 0.5 mg and 100 ml α-tocopherol.
3. The third group termed E-deficient received no α-tocopherol.
4. The E-excess group had significantly higher concentration of DNA in liver than the control or E-deficient groups.
5. On the basis of an assumed constant amount of DNA per cell, it has been possible to compute the number of cells per unit weight of tissue and per liver, as well as the cell mass.
6. It is concluded that α-tocopherol in excess amounts stimulates hyperplasia in rat liver in the young animal.

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77. Use of Massive Doses of Vitamin E in Humans and Rabbits to Reduce Blood Lipids

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Humans were given pure d-alpha tocopherol acetate orally at a level of about 40 Gm. daily for as long as a period of 1 month. No side reactions, weight gain, or loss, occurred.

Blood serum cholesterol and β-lipoprotein determinations were done on 6 male subjects. Their diets were kept constant and after the control levels were established, they ingested the pure vitamin E in 3 divided doses. Two of the patients had xanthoma tuberosum, another a hypercholesterolemic, and the others were normal controls. Some of the patients were followed for as long as 4 months. The most effective decreases in serum cholesterol were in the hypercholesterolemic patients. Two of them showed a fall of approximately 100 mg. of cholesterol after 1 month on vitamin E, with a rebound when the tocopherol was removed that almost reached pretreatment levels. Ultracentrifugation studies of the S_f values were consistent in some of the patients in that the S_f 0-10 class were most responsive to vitamin E ingestion. The fall in S_f 0-10 measured as high as 100 mg. per cent.

The rabbit experiments consisted of 3 groups of animals: group I was fed a standard rabbit chow, group II received 0.75 per cent cholesterol in the diet in peanut oil plus the chow; and group III, 0.75 per cent cholesterol plus peanut oil plus vitamin E at a level equal to 0.5 Gm. of E of body weight Kg. day. The stock α-tocopherol-cholesterol peanut oil-rabbit chow mixture was kept refrigerated, except when filling the food bins of the animals. Groups II and III showed rapid elevations of serum cholesterol over a 2 month period. Group I remained within normal limits. However, there was a distinct lag of many weeks in rise of serum cholesterol of the rabbits receiving vitamin E plus cholesterol, compared with those receiving cholesterol alone.

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Relative Rates of Liver Storage of Pure α-, β-, γ- and δ-Tocopherols in the Growing Chick

PREVIOUS work in this laboratory on the storage of vitamin E in growing chicks has shown that on y α-tocopherol was present in the liver. Since normal poultry foods often contain a high proportion of non-α-tocopherols[1,2] the ability of the chick to utilize these compounds is of importance. American workers, using the transfer of tocopherol into the egg as an index of utilization[3], found no evidence of the conversion of either γ- or δ-tocopherol to α-tocopherol, but direct measurements of liver storage were not made.

Groups of six birds of a commercial broiler strain (New Hampshire Light Sussex × White Rock) aged six weeks were placed on a purified diet low in vitamin E and depleted for a period of three weeks. Then over a period of five days they were given a series of oral doses of pure α-, β-, γ- or δ-tocopherol in ethanol–water emulsion. Two days after the final dose the birds were killed.

Blood samples were taken and whole livers were removed for tocopherol assay. Lipids were extracted using a modified form of the procedure of Swick and Baumann[4]. Saponification and removal of sterols, carotenoids and vitamin A were carried out using Brown's methods[5], and finally individual tocopherols were separated and identified by means of the two-dimensional paper chromatographic procedure of Green[2].

It was not found possible to identify any individual tocopherol in blood; but all tocopherols were found in liver with the exception of δ-tocopherol. Relative concentrations and recovery rates are given in Table 1.

The relative biological activities for α-, β-, γ- and δ-tocopherols have been quoted by Brown[5] as 100 : 30 : 20 : 1, and Ames[6] as 100 : 6 : 1 : 1 and later[7] as 100 : 33 : 1 : <1. The relative rates of liver storage quoted in Table 1 show some correlation with these values and might offer some explanation for the lower biological activities of non-α-tocopherols and especially of δ-tocopherol.

However, an additional factor in the consideration of biological activity in natural products is that of availability. Bunnell[8] has shown that the availability of α-tocopherol in lucerne to be only about 30 per cent.

Table 1

	Total dose 6 birds (mgm.)	Concentration found in fresh liver (γgm./gm.)	Total recovered in livers (mgm.)	Relative percentage recovery (α = 100)
α-Tocopherol	120	1·90 ± 0·19	0·289	100
β-Tocopherol	100	0·76 ± 0·17	0·099	41·2
γ-Tocopherol	120	0·34 ± 0·15	0·054	18·7
δ-Tocopherol	100	Nil	Nil	Nil

I am grateful to the Directors of Spillers Limited for permission to publish this communication.

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Effect of Vitamin E on Carcinogenicity of Methylcholanthrene.* (27918)

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Wheat germ oil, which is very rich in alpha tocopherol (Vit. E), is one of the dietary factors reported to affect the carcinogenic response. Jaffe(1) reduced the incidence of response to an intraperitoneal injection of methylcholanthrene more than 50% by adding wheat germ oil to the diet of mice. He did not believe that the results could be attributed to the alpha tocopherol, though this vitamin had been shown to reduce the toxic effects of methylcholanthrene. Haddow(2), as well as Cameron and Meltzer(3), have concluded that alpha tocopherol does not lessen the incidence of tumors from skin paintings with carcinogens. Rowntree *et al.* (4) fed a crude wheat germ oil to rats and guinea pigs without other carcinogens, and claimed to have produced sarcomas in the abdominal cavity with an induction period as short as 15 days. Others(5-9) were unable to confirm these findings.

There is, however, apparently no previous investigation of the effect of alpha tocopherol on subcutaneously injected methylcholanthrene. The purpose of this study was to ascertain whether oral administration of this vitamin exerted any effect on production of sarcomas by methylcholanthrene.

Experimental data. In the first experiment, 140 female mice of the C57 Leaden strain were divided into 2 equal groups, one of which had 0.67 g of alpha tocopherol added to each kilo of its Laboratory Chow diet–an increase of almost 100 times the normal content. The critical subgroup in each major group received a subcutaneous depot of methylcholanthrene in mineral oil (0.1 mg/ml) in the interscapular region.

None of the 70 control animals developed tumors in the 24 weeks of the experiment. Thirteen fibrosarcomas were found in the critical subgroup receiving a regular diet, while only 8 appeared in the critical subgroup fed a high Vit. E diet. This represents 54% and 30% respectively of those animals surviving after the appearance of the first tumor.

In the second experiment, 130 C57 Black male mice were divided into 2 equal groups, one of which had 7.0 g of alpha tocopherol added to each kilo of its Laboratory Chow diet. The two critical subgroups in each group received an interscapular subcutaneous depot of methylcholanthrene (0.1 mg/ml) dissolved either in tricaprylin or a solution of 10% Tween 60 and 90% tricaprylin.

In 26 weeks, 2 lymphomas appeared in the control animals, an incidence of 6%. The mice in the critical subgroups on a normal diet developed a total of 13 tumors (9 fibrosarcomas, 3 lymphomas, 1 hepatoma): an incidence of 33% after correction for early deaths. The critical subgroups on a high Vit. E diet had a total of 9 tumors (7 fibrosarcomas, 1 lymphoma, 1 squamous carcinoma): a corrected incidence of 21%.

Discussion and conclusions. The C57 mice have a low incidence of spontaneous tumors, but respond uniformly well to carcinogens. Age does not seem to be a prominent factor, though more primitive sarcomas are produced in very young mice(10). Mineral oil and tricaprylin, if critically purified, apparently have little or no specific influence on carcinogenesis when used as a vehicle in a subcutaneous depot. Alpha tocopherol appreciably inhibited the carcinogenic activity of methylcholanthrene in the first experiment, and failed to produce tumors in the controls. In the second experiment, which had a bascially similar plan, though differing in the sex and substrain of mice, dosage of Vit. E, and vehicle used for the carcinogen, inhibition of the carcinogenic activity of methylcholanthrene by alpha tocopherol was confirmed.

The significance of these results approximates the 93% level(11,12).

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† U. S. P. H. S. Postdoctoral Research Fellow.

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From the Physiological Institute of the Veterinaty Science College Hannover. (Director: Prof. Dr. H. HILL).

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On the Vitamin-E metabolism

The experimental production of an E-hypervitaminosis in poultry, as compared to the effect of epyphysis extracts.

Posing of the problem

The function of E-vitamin and the symptoms of E-deficiency are highly controversial; the question concerning the existence of an E-hypervitaminosis is likewise largely unclear. While α-tocopherol is regarded as a substantially non-toxic agent in animals even when higher doses are applied--the administration of several grams α-tocopherol during 2 months to rats, dogs and cats had no effect according to DEMOLE[1]--and since under normal conditions no E-hypervitaminosis need be feared in man either (BECKMANN[2]), reports on the occurence of injuries after overdoses are very rare; these reports are contradictory and refer to laboratory animals only. Accordingly, BODENHEIMER and LASH[3] found no histological changes of the testicles in the male animals after the administration of 3 - 30 mg α tocopherol to voles (microtus Güntheri); on the other hand, they found that in the ovaries follicle maturation did not occur after administration of said compound during several months. At variance form the above, daily doses of 0,5 ccm vitamin E-concentrate in olive oil caused a characteristic testicle atrophy in male rats after 7 to 8 weeks, which however was ascribed by the authors (STAHLER and collaborators) to the olive oil and not to the α-tocopherol. FECHT[5] on the other hand believes that these results involved an E-hypervitaminosis. MILOS[6] found after the continuous administration, perorally as well as parenterally, of α-tocopherol to rats during at least 4 months that the estrus phases were intensified and took an irregular course, whereby the weights of the ovaries as well as the number of mature follicles and corpora lutea decreased together with the duration of the test. No changes in the thyroid gland, hypophysis and uterus occurred during these tests, as compared to the findings of HUER[7], HAIM and SYM[8] who verified an activation of the thyroid gland after high doses of E. Reports are lacking on E-hypervitaminosis in the domestic animal.

The occurence of cycle irregularities in cows connected with the adding of vitamin E to the fodder during a prolonged period of time prompted the investigation of the fundamental question concerning the production of E-hypervitaminosis symptoms in our domestic animals.

we furthermore hoped to obtain, through the experimental production of E-hypervitaminosis, more reliable evidence on the effects of vitamin E on the endoctrine system than could so far be obtained after the application of the cutomary tocopherol doses. Our tests, the results of which are reported below, included poultry of both sexes. We set the task, as an additional goal to be reached, to test the effect of a watery epiphysis extract on the same animal material, in correlation with our vitamin E tests. The endoctrine function of the pineal gland remains controversial-- no hormone could as yet be isolated from the latter. It was found that pineal gland extract has an inhibiting effect on the male gonad of the rooster (TRAUTMANN[9]), of guinea pigs and rats (ALCOZER and GIORDANO[10]), and that it has a therapeutically important effect on the prostate carcinoma in man (as described by ALTIERI and SORRENTINO[11]), as well as on the ovaries of rats (KITEY and ALTSCHULE[12], JOSCHLE[13]) and rabbits (JAILE[14]). These findings are opposed by opinions according to which no effect on the endoctrine system occurs (SEIDEL, among others[15]). The results of TRAUTMAN[9] in particular are neverthless especially important; he interprets the effect of the epyphysis as antigonadotropic, because the location of the attack is presumably the same as with Vitamin E, namely: the gonads or the organ respectively, which is superimposed to them, i.e. the hypophyisis .*) Therefore a watery bovine epiphysis extract named Glapenin*) was included in our Vitamin E tests.

Test system of 1st test series.

During the 1st test series, 14 animals (Group I) (7♂ - Group I a, 7♀ = Group I b) from a total of 18 chicks from a crossbreed, of both sexes (9 males, 9 females) which were 3 weeks old when the tests started, received, besides their customary feed ad libitum, DL -α- tocopheralacetate[1]) during 7 weeks, administered parenterally; accordingly each test animal received twice per week during the following 3 weeks 200 mg/week. The total dose per animal therefore amounted to 2000 mg. The remaining 4 animals served as controls (Group II) (2♂ = II a, 2♀ = II b). Body weight and in in male animals , the development of secondary sexual characteristic, crest , wattle, were currently checked. Furthermore, attention was paid to disease symptoms. After the termination of the test all animals were killed; their testicles, ovaries and the other endoctrine organs were weighed and histologically examined.[2])

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*) Preparation of the business firm : Hormon-Chemie, Munich.

1) water-soluble preparation of the business firm Hoffmann-La-Roche.

2) The histological examination was carried out by the Pathology Institute of the College for Veterinary Science , Hannover, for which thanks are expressed.

Test system of 2nd test series.

During the 2nd test series in the course of 10 weeks 28 female and 28 male chicks of the white Leghorn breed, approx. 2 weeks old, were assigned to 4 groups including 14 animals each, half of which were young roosters and young hens respectively, in each case.

The animals of Group 1 (1 a- 7 male chicks; 1 b = 7 female chicks) received twice weekly 1ml each of Glanepin /animal subcutaneously, besides feed ad libitum; the feed was the same for all groups.

Group 2 included 7 male (2 a) and 7 female (2 b) chicks; these received, (besides the same dosage of Glanepin as for Group 1) additionally during 4 weeks, administered twice weekly, 100 mg each, and during the following 6 weeks twice weekly 200 mg each of DL-α- tocopherolacetate, applied parenterally. The total dose per animal amounted to 20 ml Glanepin plus 3000 mg Vitamin E.

The 14 animals which formed Group 3 (half of them male (3 a), and half of them female (3 b)) served as control animals without specific feed additives.

From the 14 animals included in Group 4 12 animals succumbed within the first 4 weeks of life alone, evidently as a result of the supplied Vitamin E doses; therefore only 2 animals (No. 47 and No. 58) survived a test period of 8 weeks. They received DL-α-tocopherolacetate in the same way and to the same amount as the animals in Group 2, i.e. each animal received a total of 3000 mg Vitamin E. Since the effect of the E vitamin was definitely evident from the results of the 1st test series in case of 14 animals, no more animals were added to this group in the course of the test.

Table 1. Weekly body weights in g of chicks (crossbreed) under the effect of DL-α-tocopherol.

Tiernummer	Gruppe Ia (♂ Vitamin E)							
	64	65	66	67	68	69	70	Mittel
3. Lebenswoche	247	167	181	187	171	207	200	194
4. "	340	243	267	269	247	312	319	285
5. "	440	354	372	380	337	461	458	400
6. "	521	425	423	500	407	597	595	495
7. "	642	473	506	660	514	710	759	609
8. "	716	567	524	754	531	656	964	673
9. "	675	586	530	688	550	697	999	675
10. "	695	607	553	695	560	718	1040	695

Tiernummer	Gruppe IIa (♂ Kontrollen)		
	78	79	Mittel
3. Lebenswoche	210	232	221
4. "	319	340	330
5. "	463	475	469
6. "	624	658	641
7. "	760	830	795
8. "	966	1092	1029
9. "	990	1147	1068
10. "	1076	1296	1186

Tiernummer	Gruppe Ib (♀ Vitamin E)							
	71	72	73	74	75	76	77	Mittel
3. Lebenswoche	173	156	168	196	157	191	151	170
4. "	242	225	229	206	220	285	280	241
5. "	351	302	315	395	309	412	289	382
6. "	434	351	357	483	376	548	325	410
7. "	514	388	416	570	439	692	345	480
8. "	621	464	467	698	501	791	330	553
9. "	648	496	483	718	507	785	359	570
10. "	683	550	434	770	525	773	360	585

Tiernummer	Gruppe IIb (♀ Kontrollen)		
	80	81	Mittel
3. Lebenswoche	173	173	173
4. "	279	258	269
5. "	406	368	387
6. "	546	465	506
7. "	691	583	637
8. "	775	783	779
9. "	825	810	818
10. "	906	847	876

1) No. of animal
2) Group I a (♂ - Vitamin E.)
3) Group II a (♂ controls)
4) Average
5) Week of life
6) No. of animal
7) Group I b (♀ Vitamin E)
8) Group II b (♀ controls)
9) Average
10) Week of life

After 10 weeks of testing and body weight checking all animals were killed; crest, testicles and ovaries were weighed and the gonades were histologically examined.

Results of the 1st test series.

The results of the body weight development, shown on Table I and Illustration 1, indicate that the growth of the male test- and control animals is somewhat faster; furthermore, the results indicate that animals of both sexes treated with α-tocopherol definitely show inhibited growth. As evident from Ill. 1, said inhibition intensifies in the course of the test period. In the 8th to 10th week of life the weight increase of roosters and hens is minor; in sporadic cases the weight increase even comes to a total standstill. Differences in the body weight increase of the animals in Group I as compared to Group II are confirmed.

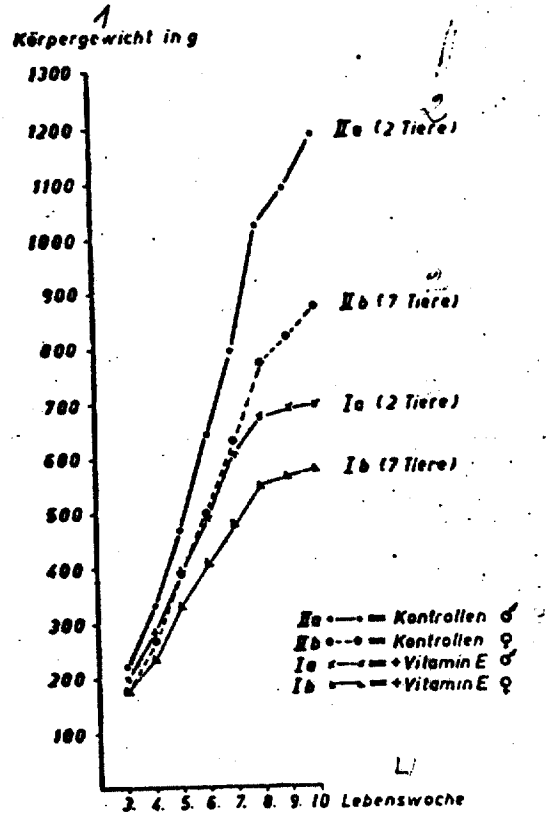


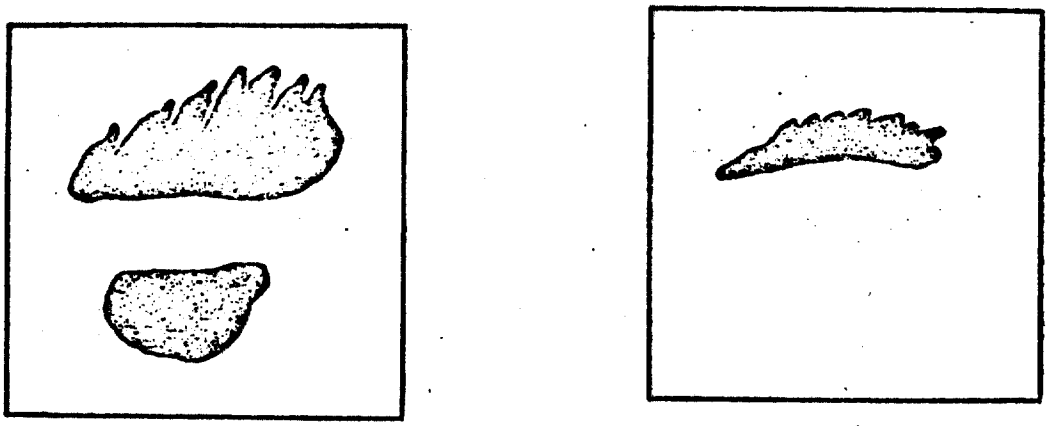
Illustration 1. Average weight development of chicks (crossbreed) under the influence of DL-α- tocopherol.

1) Body weight in g
2) . . animals
3) . . . controls
4) . . . week of life.

we believed that we could dispense with a number of control animals in this test series equal to the number of the vitamin E-animals, in view of the high number of control animals of the white Leghorn breed which received the same feed in the course of the 2nd test series.

While no disease symptoms whatsoever were observed in the control animals and while the male (control) animals showd all indications of beginning sexual maturity during the last weeks, increasingly evident symptoms of debility, lack of appetite as well as dull plumage and an inclination to moult were detectable in the animals treated with vitamin E. Towards the end of the test the appearance and behavior of the young roosters did not differ from the appearance and behavior of the young hens. In particular, no definite difference was evident in the development of the crest and wattle of the test animals of both sexes, as compared to the development of these secondary sexual characteristics in the young control roosters. Illustration 2 shows a comparison between the size of a wattle and crest of a control animal and of a young rooster of the same age which was treated with vitamin E. The wattles of the latter animal could practically not be measured.

Illustration 2. Surface of crest and wattle from a control rooster (No. 79) and from a test rooster (No. 66); approximate age:10 weeks.



a

a

Crest and wattle of a control rooster; age: 10 weeks.

b

b

Crest and a rooster treated with high doses of DL-α- tocopherolacetat age: 10 weeks.

The crest weights of all animals (Table 2) show differences related to sex and to applications of Vitamin E in male animals, with a statistical verification of $p < 0,05$. The testicles of the test animals are far smaller in size as compared to those of the control animals, as evident from the same Table and from III.3. The verification is given with $p < 0,01$. Reference is furthermore made to the correlation between testicle weight and crest weight, insofar as a higher testicle weight generally also coincides with a corresponding increase of the crest size.

The expected histological diagnosis likewise corresponds to said testicle weight differences: the testicles of the test animals show no indications whatsoever of sexual maturity. (Ill. 4 to 7).

Table.2. Organ weights and histological diagnosis for Vitamin E-animals and for controls (in mg).

Nr.	Kamm	Hoden	Ovar	Histologischer Befund
Vitamin-E-Tiere				
64	803	88		Epithel ein- oder zweireihig.
65	162	56		Spermien nicht nachweisbar.
66	153	34		Geringgradige Fibrose.
67	517	72		
68	624	73		
69	538	48		
70	591	79		
71	91		295	
72	67		195	
73	71		180	O. B.
74	47		271	
75	90		154	
76	67		254	
77	30		195	
Kontrollen				
78	720	199		Epithel allgemein mehrreihig.
79	3601	313		Spermien i. einigen Kanälchen
80	175		219	
81	249		248	O. B.

1) Vitamin E-animals.
2) Crest
3) Testicles
4) Ovaries
5) Histological diagnosis
6) Epithelium in one or double row
7) Sperms not detectable
8) Minor fibrosis
9) No diagnosis
10) Controls
11) Epithelium in all rows
12) Sperm in some ducts

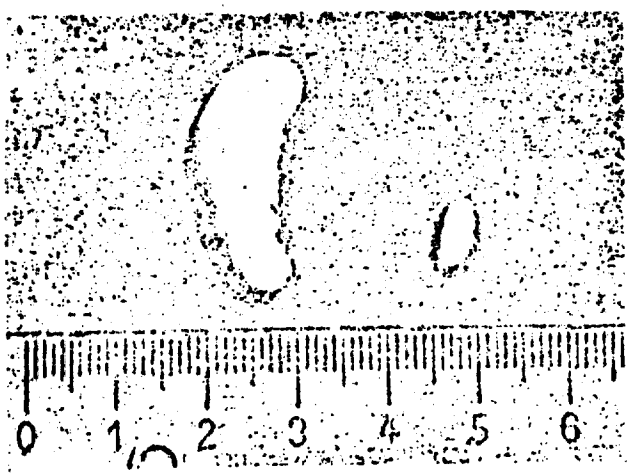


Illustration 3. Testicles of a control rooster (left) and of a rooster (right) treated with DL- - tocopherolacetate age of both: 10 weeks.

Accordingly, the multiple-row epithelium of the small testicle ducts and the mature sperm are missing, as compared to the histological evidence of spermiogenesis in the gonades of the control roosters (Ill. 4 to 7).

Illustration 4 and5. Small testicle ducts of a control animal. Age: 10 weeks.

Illustration 4.
Dye: hematoxyline-eosin.

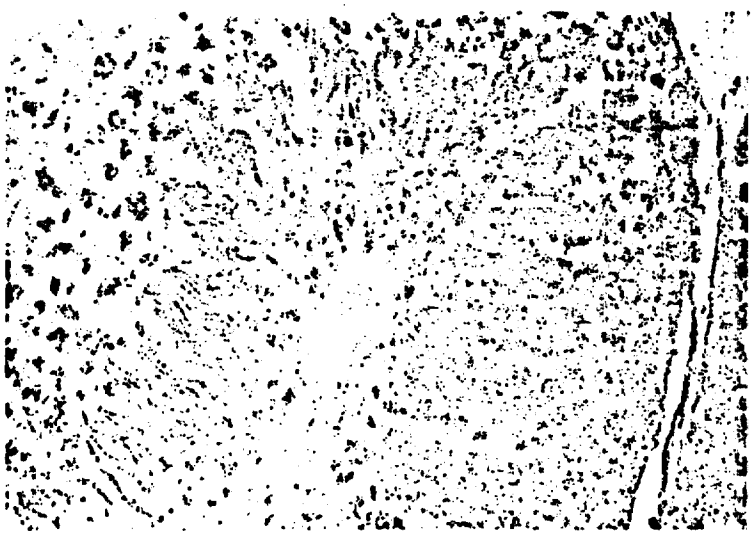


Illustration 5.
Dye: hematoxyline-eosin.

Illustration 6 and 7. Small testicle ducts of a Vitamin E-animal. Age: 10 weeks.

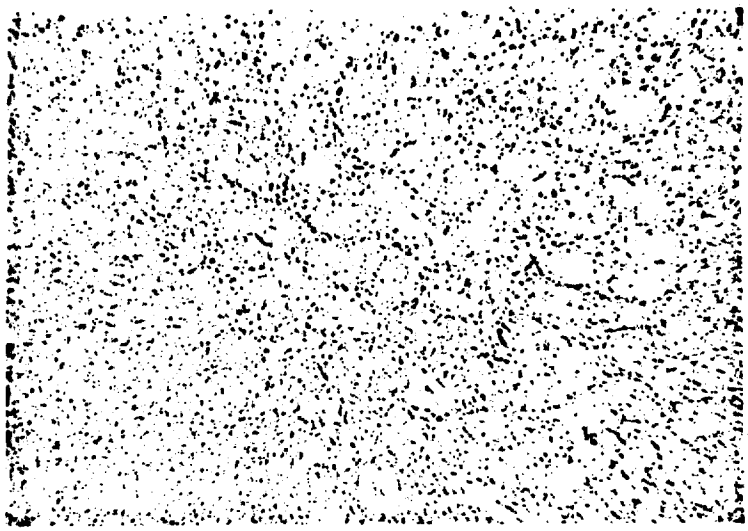


Illustration 6.

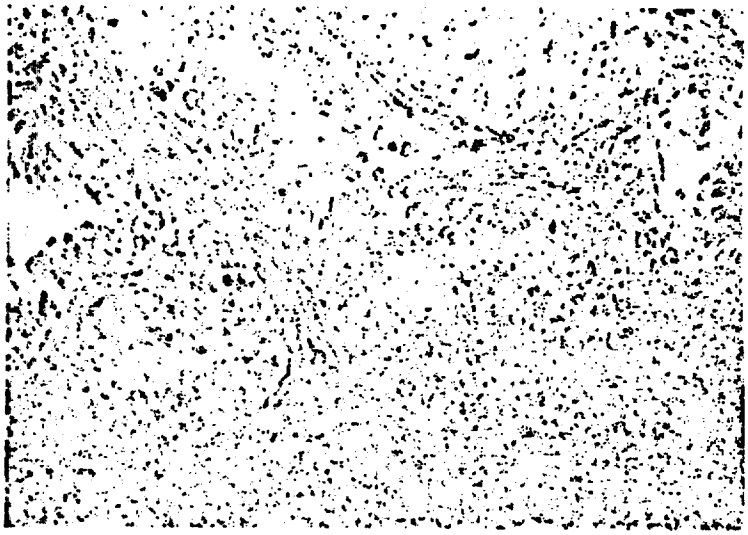


Illustration 7.

The weights and the histological ovary diagnoses for the E-animals, on the other hand, did not demonstrate any changes in this organ, as compared to (the ovaries of) the normal animal. No confirmed differences under the effect of the vitamin resulted in the other endocrine organs, the hyroid gland, hypophysis and suprarenal glands.

Results of the 2nd test series.

As evident from Table 3, the course of general body growth, recognizable from the development of the body weight, is most obviously inhibited in case of the two Vitamin E-animals of Group 4, and then also in the animals treated with α-tocopherol and Glanepin (Group 2), as compared to the control animals of Group 3. This applies equally to the female and the male sex, whereby the fact has to be noted that the weight increase of the male animals is greater in each case than the weight in the young hens from the same animal group. As for the test animals of the 1st test series, the growth increase is less with advancing age and with higher vitamin E doses respectively. No delayed body weight development, caused by Glanepin, occurs in the male animals and has not been confirmed in the hens either.

Table 4 shows that underdeveloped testicles with disorders of the spermiogenesis were found in all male animals supplied with high doses of vitamin E (Group 2 a and 4 a). The crest weight was correspondingly minor as compared to the control animals (Group 3 a) with much higher (up to 10 times and more) crest- and testicle weight, indicating sexual maturity, which could be determined in each case by displaying mature sperms. Organ weights and histological findings for the young roosters treated with epyphysis extract were not uniform, insofar as the data showed sexually mature animals (2 animals) as well as underdeveloped animals (5 animals) in the course of 10 weeks with continuous administration of Glanepin. The body weight increase of the two animals with efficiently functioning testicles was 200 g more than in case of the remaining 5 animals.

Weights and tissue structure of the ovaries (see Table 4), as well as the other examined glands did not show any specific characteristics which could safely be attributed to the effect of Glanepin or Vitamin E.

Discussion of the results.

The diagnosis of well-defined growth inhibition in chicks of both sexes, as well as of the primary and secondary sexual characteristics respectively, of the testicles, crest and wattles in young roosters of two different breeds may be considered as caused by high doses of DL-α-tocopherolacetate. Of course the question is raised: in what way does this vitamin interfere with the sexual development, which interference is obvious in the male animal only? Our results undoubtedly lead to the assumption that an effect counteracting the gonadotropic hormones of the hypophysis front lobe is present, if the male gonad is involved. The interpretation of the findings will be possible after additional investigations only.

It seems that the effect on Glanepin on the male gonad is similar to the effect of high Vitamin E doses; said effect (of Glanepin) however, is less strong in case of our developing animals with the doses used by us; said effect cannot be confirmed in all cases.

At variance from Vitamin E, Glanepin has no effect on the body weight. -At this time, a new test series is in course to determine how far the numerous injections as such affect the general development of the small and young animals. The conclusion can be reached from the fact that findings vary according to sex, as well as from the fact that no inhibiting reaction affecting the body weight is present in the animals of the 2nd test series injected with Glanepin, that possible damage caused by the application method is not decisive for the results. The possible involvement of the solvent used for the vitamin E is likewise under investigation at this time.

Table 3. Weekly body weights of chicks (white Leghorn) under the influence of DL-α-tocopherol and Glanepin.

Tiernummer	Gruppe 1a (♂-Glanepin) 31	32	33	34	35	36	37	Mittel
2. Lebenswoche	62	83	69	83	74	83	68	74
3. "	116	153	136	144	138	141	114	134
4. "	194	224	233	223	202	233	182	213
5. "	315	573	343	330	309	362	276	329
6. "	394	496	472	425	415	494	355	435
7. "	480	583	545	475	482	600	435	500
8. "	586	658	651	555	581	668	519	602
9. "	627	667	739	646	628	750	577	662
10. "	596	757	827	733	737	908	681	748
11. "	708	845	937	801	805	963	774	833
12. "	872	957	1096	920	925	1087	890	963
13. "	1065	1155	1280	1057	1096	1330	—	1163

Tiernummer	Gruppe 1b (♀-Glanepin) 38	39	40	41	42	43	44	Mittel
2. Lebenswoche	76	58	57	59	58	65	67	62
3. "	127	97	101	108	102	108	109	107
4. "	202	151	152	176	147	171	167	166
5. "	293	225	224	259	213	255	258	246
6. "	374	295	287	407	269	327	339	328
7. "	442	342	337	404	349	384	388	363
8. "	465	341	358	446	377	429	440	408
9. "	494	344	385	497	412	465	478	439
10. "	568	385	422	558	474	516	541	494
11. "	638	402	475	641	539	561	611	552
12. "	698	448	550	716	622	635	693	623
13. "	853	570	685	828	—	778	805	753

Tiernummer	Gruppe 2a (♂-Vit.-E-Glanepin) 16	21	24	25	27	28	29	Mittel
2. Lebenswoche	58	64	69	63	60	74	64	64
3. "	125	102	119	101	133	102	120	114
4. "	195	153	168	152	196	174	185	174
5. "	339	213	278	231	319	273	292	277
6. "	460	304	376	313	422	377	375	375
7. "	514	354	459	413	494	443	418	442
8. "	568	392	503	466	587	440	444	485
9. "	608	426	535	497	641	488	487	526
10. "	657	474	575	542	667	505	520	562
11. "	691	533	630	593	784	577	595	629
12. "	730	550	702	641	742	657	651	667
13. "	782	—	803	736	852	735	735	773

Tiernummer	Gruppe 2b (♀-Vit.-E-Glanepin) 17	18	19	20	22	23	26	Mittel
2. Lebenswoche	60	66	56	60	64	54	60	60
3. "	98	113	102	100	101	92	101	101
4. "	158	191	158	156	155	152	161	161
5. "	250	290	238	244	231	200	241	250
6. "	324	383	319	335	310	359	319	335
7. "	379	391	383	371	362	398	380	380
8. "	418	439	402	393	391	474	414	418
9. "	434	492	453	385	444	513	453	453
10. "	446	493	473	437	460	526	476	473
11. "	450	528	512	533	491	606	520	520
12. "	488	537	503	550	647	702	550	537
13. "	498	570	500	541	490	—	560	543

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Table 3 (continued)

Tiernummer	Gruppe 3a (♂-Kontrollen) 1	2	3	4	5	6	7	Mittel	Gruppe 3b (♀-Kontrollen) 8	9	10	11	12	13	14	Mittel
2. Lebenswoche	70	69	67	79	63	71	69	70	62	70	79	70	74	70	69	70
3. "	124	130	114	146	128	126	129	128	120	128	147	115	139	129	121	126
4. "	195	195	197	160	195	194	196	190	185	186	196	166	196	195	182	186
5. "	328	335	318	343	343	330	366	338	281	297	341	268	337	302	255	297
6. "	437	430	430	433	474	442	493	448	354	379	424	323	422	386	365	379
7. "	552	538	510	513	616	545	605	554	428	451	497	391	509	447	435	451
8. "	606	587	570	588	726	626	637	620	450	484	548	415	563	421	511	484
9. "	627	709	597	688	802	672	739	691	456	516	580	432	568	534	527	516
10. "	690	748	680	769	895	740	793	759	456	570	621	492	642	640	574	570
11. "	732	814	725	839	971	835	850	824	502	607	668	534	665	630	643	607
12. "	848	909	820	955	1079	960	950	932	559	689	765	612	740	733	730	689
13. "	1034	1090	981	1129	1275	1149	—	1110	672	815	919	747	—	853	886	815

Tiernummer	Gruppe 4a (♂-Vit. E) 47	Gruppe 4b (♀-Vit. E) 58
2. Lebenswoche	75	68
3. "	95	92
4. "	185	165
5. "	297	256
6. "	390	335
7. "	494	409
8. "	448	460
9. "	502	430
10. "	487	418
11. "	511	449
12. "	534	469

Table 4. Organ weights and biological findings for test animals and controls (in mg).

	Nr.	Kamm	Hoden	Ovar	Histologischer Befund
Gruppe 1a (Glanepin)	31	9454	556		Hochgradige Störung der Spermiogenese
	32	18046	491		
	33	28665	4500		Normale Spermiogenese
	34	13313	1298		Störung d. Spermiogenese
	35	10360	377		Hochgr. Störung der Sp.
	36	31425	6761		Normale Spermiogenese
	37	9511	210		Hochgr. Störung der Sp.
Gruppe 1b (Glanepin)	38	296		233	O. B.
	39	109		143	"
	40	551		226	"
	41	485		251	"
	42	156		200	"
	43	415		221	"
	44	314		230	"
Gruppe 2a (Glanepin + α-Tokoph.)	16	5506	131		Hochgr. Störung der Sp.
	21	1314	53		"
	24	4060	170		"
	25	2838	97		"
	27	1918	125		"
	28	785	92		"
	29	1933	111		"
Gruppe 2b (Glanepin + α-Tokoph.)	17	105		142	O. B.
	18	139		150	"
	19	104		185	"
	20	101		158	"
	22	73		217	"
	23	226		218	"
	26	110		147	"
Gruppe 3a (Kontrollen)	2	25657	3006		Normale Spermiogenese
	3	8850	659		"
	4	13779	1862		"
	5	25618	5302		"
	6	24812	2020		"
	7	23461	2854		"
Gruppe 3b (Kontrollen)	8	217		180	O. B.
	9	236		255	"
	10	546		381	"
	11	249		276	"
	12	214		167	"
	13	230		262	"
	14	276		265	"
Gruppe 4a	47	1314	53		Hochgr. Störung der Sp.
Gruppe 4b (α-Tokoph.)	56	100		138	O. B.

1) No.
2) Crest
3) Testicles
4) Ovary
5) Histological finding
6) Group
7) Control

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Am. J. Clin. Nutr. 5: 597-600, 1957

Tocopherol Excess in Man

CREATINURIA ASSOCIATED WITH PROLONGED INGESTION

By Robert W. Hillman, M.D.*

An essential role for vitamin E in human metabolism has yet to be established. However, its well documented indispensability to certain functions in experimental animals, and its seeming relationship to metabolic abnormalities described in patients apparently depleted of this substance suggest that vitamin E is probably also required by man.

Although tocopherol therapy generally fails to benefit patients with muscular dystrophy, the creatinuria that accompanies the reversible muscular disorders characteristic of vitamin E deficiency in other species may have a clinical counterpart. In a recently reported case of fatal biliary cirrhosis,[1] creatinuria and pentosuria were associated with an absence of measurable tocopherols in the blood serum, and were temporarily alleviated by tocopherol administration. Similarly, vitamin E therapy has inhibited creatinuria in infants with cystic fibrosis of the pancreas and biliary atresia.[2]

The present report describes an instance in which the experimental ingestion of an excess of tocopherol over a period of three months by an apparently normal subject was associated with a transitory creatinuria in the absence of other significant adverse clinical or laboratory effects.

CASE REPORT

A 41-year old physician ingested a total of 296 g of alpha tocopherol over a period of 93 days, in the following dosage: 2 g daily for 37 days; 4 g daily for 55 days; and 2 g on the final day. Two preparations were employed: Ephynal Acetate*† for the first 80 days; and Aquasol E*‡ for the last 13 days. Usually the vitamin was taken in two daily doses about eight hours apart, but there was no rigid adherence to this routine or to any fixed dietary regimen. There was no appreciable change in physical activity during the test period, nor were any significant intercurrent infections incurred.

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RESULTS

The principal laboratory findings are indicated in the accompanying table and figure.

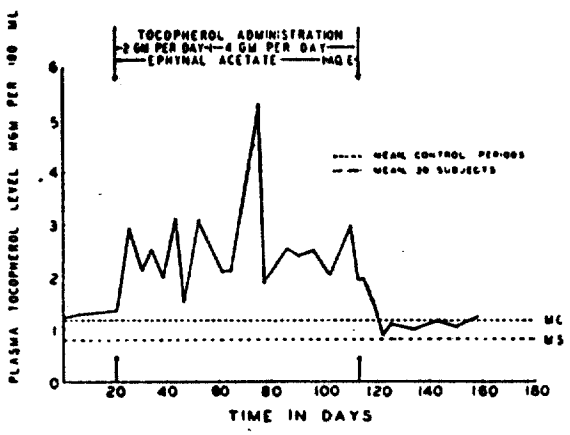

Fig. 1. Blood plasma tocopherol response to ingested vitamin E: 296 g DL-alpha tocopheryl acetate in 93 days.

The plasma tocopherol concentration, measured by a macro adaptation of the method of Quaife *et al.*,[3] showed a rapid initial rise followed by a variable plateau without overall increment, which was maintained throughout most of the test period at approximately twice

† Ephynal Acetate, Roche, supplied in part through the courtesy of Dr. S. Evert Svenson, Hoffman-La Roche, Inc., Nutley, N. J.

‡ Aquasol E, U. S. Vitamin Corporation.

TABLE I

Plasma Tocopherol Concentration and 24-hour Urinary Excretion of Creatine and Creatinine Before, During, and Following Excess Tocopherol Ingestion

Observation day	Test period day	Plasma tocopherols*	Urine creatine†	Urine creatinine†
1	−19	1.26		
7	−13	1.39		
19	−1	—	0	1,704
20	1	1.41		
25	6	2.91		
29	10	2.25		
32	13	2.53		
36	17	2.01		
40	21	3.13		
43	24	1.53		
47	28	3.09		
60	41	2.18		
64	45	2.19		
68	49	5.39		
71	52	1.79		
83	64	2.51		
88	69	2.39		
92	73	2.41		
103	84	2.98	151.5	1,313
109	90	1.91		
110	91	—	143	1,324
111	92	1.94		
113	+1	1.53		
117	+5	0.81		
120	+8	1.10		
124	+12	—	0	1,322
130	+18	1.03		
139	+27	1.18	0	1,379
155	+43	1.03		
162	+50	1.24		
169	+57	0.76		
186	+74	1.29		
195	+83	1.29		
228	+116	1.40		

* Mg per 100 ml.

† Mg per 24 hours.

the usual concentration.* It returned rapidly to the pretest range when the tocopherol was discontinued. The mean fasting concentration of 2.26 ± 0.84 mg per 100 ml differed significantly (P less than 0.001) from the mean level of 1.15 ± 0.36 per 100 ml before and after the test period.† The fasting concentration was not appreciably higher on the daily dose of 1 than of 2 g, nor did the substitution of the aqueous dispersion for the nonwater-miscible preparation have any apparent added effect. Tocopherol could not be detected in the urine at times of maximum plasma concentration or over spans of several hours following ingestion of this substance.

A significant creatinuria was noted during the test period. On the day before initiation of the vitamin regimen, creatine was absent from a 24-hour specimen. On the 84th and 91st days of the experiment, the 24-hour excretion totals were 151.5 and 143 mg respectively. Twelve, and again, 27 days after the tocopherol was withheld, creatine could not be detected in 24-hour samples.

Creatinine excretion was questionably altered by the tocopherol ingestion. The output in the 24-hour period before the start of the test was greater by approximately 20 to 25 per cent than quantities measured on two occasions during, and two following, the experimental period. Except for a slight increase 27 days after the preparation was discontinued, these four readings were virtually identical. The excretion of 17-ketosteroids seemed unaffected by the tocopherol administration being 11.3 mg per 24 hours on the day before, and 11.9 mg on the 84th day of the tocopherol regimen. Blood cholesterol, carotene and vitamin A levels, the serum bilirubin level, cephalin flocculation, thymol turbidity, and the prothombin, bleeding and coagulation times showed no significant changes during the test period. The Harvard two-step test for physical fitness, Master two-step test, electrocardiogram and ballistocardiogram were likewise unaffected. Biopsy of the deltoid muscle at the end of the

* Repeated absorption tests showed considerable variation, with no consistent plasma response pattern. In general, following ingestion of amounts up to 4 g in a single dose, plasma tocopherol levels showed only small, gradual changes. Apart from the single fasting level of 5.39 mg per 100 ml, a concentration in excess of 4.0 mg per 100 ml was noted in only one instance, six hours after the ingestion of 4 g of tocopherol.

† The mean plasma level of 1.16 ± 0.39 mg per 100 ml before that period did not differ significantly from the level of 1.12 ± 0.20 mg per 100 ml following the experiment. However, the mean fasting level for this subject (62 readings before and after the test period) of 1.15 ± 0.37 mg differed significantly (P less than 0.0001) from the mean fasting level of 30 other subjects (72 readings) of 0.81 ± 0.26 mg.

experimental period revealed no deviation from the normal.

Clinically, no abnormalities were noted that could be attributed unequivocally to the large doses of tocopherol consumed. However, chapping cheilosis and angular stomatitis appeared early and persisted variably, but without progression, throughout the ingestion period. Gastrointestinal disturbances were frequent, especially during the final month, when abdominal distress and diarrhea with tenesmus were pronounced. Vague generalized muscle weakness and increased fatigability were experienced toward the end of the test period, but these wholly subjective complaints could not easily be evaluated. The untoward clinical manifestations disappeared within two weeks after the tocopherol was discontinued.

DISCUSSION

The average plasma concentration maintained in this case exceeded the usually accepted range of normal, but was lower than maximum levels reported with much smaller doses.[4–7] Higher levels have also been noted in the last trimester of pregnancy,[8,9] and in certain pathologic states without apparent excess ingestion of this substance.[9–11] The type of preparations employed may have been a limiting factor, and large amounts may have been lost in the stool.[5,12–14] Nevertheless, the lack of progressive increment in the blood, and, as in most other reported instances,[12,15,16] the absence of vitamin E from the urine, suggest that mechanisms other than those of absorption and elimination are involved in regulating plasma tocopherol concentration. Blood levels are temporarily depressed in a number of conditions not affecting intestinal fat transfer,[7,10,17,18] and so-called ceiling effects have also been noted by other observers.[2,18]

Creatinuria, as a phenomenon of tocopherol excess, is unexpected and not readily explained. Vitamin E reportedly influences pituitary-adrenal function and questionably affects the production of 17-ketosteroids.[19–22] Yet the unchanged steroid excretion in the present instance, although based on limited data, suggests that altered creatine metabolism does not result from modified adrenal or testicular activity.*

Tocopherol toxicity is readily produced in experimental animals. Although usually stated not to occur in man,[23] untoward symptoms have been reported on doses of 300 to 1,500 mg per day.[6,24–26] While a different dosage and/or different subject might have yielded higher plasma levels, with or without clinical toxicity, the absence of definite adverse changes in this case suggests an appreciable tolerance for tocopherol. Creatinuria, conceivably, may represent an early, subtle index of induced metabolic malfunction.

SUMMARY

Ingestion of 296 g of alpha tocopherol by a normal adult male over a period of 93 days resulted in a sustained average plasma tocopherol concentration of 2.26 ± 0.86 mg per 100 ml. This was statistically significant and approximately twice the control level of 1.15 ± 0.37 mg per 100 ml. A significant transitory creatinuria occurred during the test period, but there was no apparent change in the excretion of creatinine or of 17-ketosteroids.

There were no unequivocal signs of clinical toxicity. Exercise tolerance seemed unaffected. The electrocardiogram, ballistocardiogram, serum cholesterol, liver function and blood coagulation studies, and a muscle biopsy showed no deviation from the normal.

Creatinuria may be an early manifestation of an adverse metabolic effect induced by vitamin E excess.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. Philip Cabaud for the creatine, creatinine, cholesterol, liver function and blood coagulation studies; to Dr. David Kydd for the 17-ketosteroid determinations; to Drs. William Noble and Helen Paulino for the Master two-step, electrocardiogram and ballistocardiogram; to Dr. Joseph Whelan for performing, and to Dr. J. Arnold De Veer for interpreting the muscle biopsy; and to Miss Marie Leogrande, who performed all of the vitamin determinations.

* This relationship is being investigated further. Preliminary observations suggest that a transitory creatinuria may, in some instances, be induced by a single large dose (4 g) of tocopherol, without prolonged ingestion of excessive amounts.

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THE BIOCHEMISTRY OF THE THYROID FUNCTION VII /1/: Indications of E-Hypervitaminosis in Animal Experiments

Huter, F. (Dept. of Biochemistry, Institute for Chemotherapy Research "Georg-Speyer-Haus", Frankfurt/M.). Z. Naturforschung 2b 414-419 (1947).

Dedicated to Prof. Dr. Richard Otto, Privy Councilor, on the occasion of his 75th birthday.

Summary:

The feeding of food high in Vit. E to young male rabbits leads after several weeks to pathological phenomena in the thyroid gland (SD). The 3 symptoms of the E-hypervitaminosis formed in this manner in the internally secreting organ are: 1. Abnormal growth in volume and weight; 2. Absence of or deficiency in iodine, and, 3. Histological changes (enhanced epithelial growth). The addition of Vit. E does not neutralize the harmful effect on the thyroid of goiter-generating cabbage feed. Daily addition of alpha-tocopherol does not inhibit the goiter causing thiouracil effect in rabbits, but acts in the same sense.

The importance of tocopherol in the growth, metabolism and the prevention of disturbances in the body is insufficiently known. Fundamental metabolic determinations by Verzar /2/ & al. on rats provided merely an indication of the relationship between nutrition and thyroid activity. Later, the effect of Vit. E-containing wheat germ oil activating thyroid action in white mice and rats was histologically detected /3/. Then, experimental investigations on animals concerning the effect of Vit. E in the pure state on the thyroid showed merely that tocopherol additions act on the thyroid by way of the ovary /4/.

H. Winkler demonstrated on humans that excess of Vit. E lead to restricted excretion of follicular hormone, progestrone and prolan. The concept that the hormone production of the thyroid gland would also be reduced by chronic, excessive administration of the E factor thus appeared to be logical. Based on this consideration, the work represents an attempt to detect relationships between the thyroid gland of rabbits and tocopherol. In addition, it was sought to determine whether the goiter-generating effect of cabbage low in ascorbic acid and of syntheticthiourical can be inhibited or compensated with an artifically raised Vit. E level in the animal body. /6/.

The experiments indicate the following: 5.5 to 12.5 mg tocopherol acetate per kg of body weight, daily administrations exceeding the sensitivity threshold, reveal a significant difference in after 1-3 months between males and females in otherwise normally fed rabbits with respect to the thyroid function. In female animals, highly active thyroids are produced, but with normal iodine protein contents, while male rabbits exhibit abnormal changes in the thyroid following excessive Vit. E doses, e.g. iodine deficiency, so that the designation of a state of E-hypervitaminosis is justified. In the latter case, the endocrine organ is hyperplastic, mostly free of iodine, occasionally increased in size, thus indicating functional damage to the thyreoids.

In addition, in some animal experiments the observation was made that SD distrubances generated with white cabbage or thiopyrimidine are not prevented by tocopherol excesses.

In 10 experimental animals fed with alpha-tocopherol /7/, gravimetric, histological and chemical investigations indicated a deviation in the SD from the normal organ. Thyreodia changes in the present investigation, caused experimentally by Vit. E, are probably hyperthyreoses. Thus, Hain & Sym /8/ report on treatments with Vit. E of agitation during menopause, some of which lead to hyperthyreodism.

Even in short-term experiments, 8 mg tocopherol acetate/kg of rabbit (No. 9002 in Table) resulted in moderate hypertrophia and cell hyperplasia /9/; an SD weight of 64 mg/kg body weight was found. The order of magnitude of the reduced normal thyroid weight of rabbits is given as 40-60 mg/kg of body weight.

In about twice the experimental period given above, a thyroid gland with weak cell propagation was produced in Animal No. 472 by the daily administration of 20 mg tocopherol acetate. The gland exhibited a reduced iodine content (4.5 mg%); the normal value is 10-20 mg%. This less than extreme result is caused by the fact that the Vit. E addition was discontinued one day before the last day of the experiment so that the gland was able to store iodine protein. It is therefore likely that discontinuation of tocopherol would rapidly reverse the pathological state of the SD. A thyroid gland increased in size more than three times (red. wieght 226 mg/kg) developed in a slow experiment in Rabbit No. 470, fed daily with 10 mg tocopherol. The histological section of the thyreodia is shown in Fig. 1. Minimum glycogen values and the restless behavior of the animal are additional indications of a strong hyperfunction of SD.

Rabbit No. 494 received 20 mg Vit. E/day for 3 months. The iodine analysis of the SD resulted in a subnormal level. The low halogen content was again possibly the result of the discontinuation of the tocopherol treatment, as in the case of Rabbit No. 472.

Daily administration of 20 mg Vit. E to Rabbit No. 478 produced no change in normal appearance, except for a moderate activation of the thyroid gland.

The administration of 20 mg tocopherol acetate/day to the female animal 487 resulted in a strong colloid secretion (except iodine protein); whish is seen from the low SD fresh weight (0.09 g) and the high value of the iodine (18.47 mg%).

Per os applications of 1.34 g Vit. E in daily doses of 20 mg had a favorable effect on the thyroid of Rabbit 483, in which a nautral goiterous state was induced through white cabbage feed. In contrast to earlier experimen /10/ without excessive Vit. E additions to the food, the thyreoids now

showed normal iodine values. For the hyperplastic structure of the thyroid gland, thioglicoside, which is not known with respect to chemical structure, must be held responsible. Experiment No. 483 deserves further attention, because abundant milk secretion and three times repeated nest building with hair plicking indicated false pregnancy. This nymphomanic extended heat state of rabbits is probably caused by the extensive production of adequate amounts of follicle hormone /11/.

FIGURE 1. Thyroid Gland with Indications of Strongly Increased Activity and Colloid Flush; Epithelial Tumor. Male Rabbit No. 470. (190x)

FIGURE 2. Hyperplastic, Active Thryoid Gland with Excretion. Female Rabbit No. 474. (190x)

FIGURE 3. Boundary Section of the Damaged Thyroid Gland of Rabbit No. 492; Thiouracil Animal Fed with Vitamin E. (190x)

A parallel experiment performed with Rabbit 484 had similar results with respect to the effect on SD, except that the flushing of the gland in the hyperplasia phase occurred further toward the front. The cause of the loss of weight of the animal was not investigated.

In contract to the experiments mentioned in the introduction, the female rabbit 474 displayed active but otherwise normal threoidea. Fig. 2 presents a section of the gland. It is possible that female animals require a greater Vit. E load than males for the development of SD hypertrophy, or it does not occur at all. In the case of this young animal, the uterus hypertrophy is noteworthy.

Rabbit 492 received, in addition to the Vit. E supplement of the food, 17 thiouracil injections under the skin. On each occasion, 55 mg cyclic thiourea, finely distributed in 2 cm^3 1% sodium bicarbonate solution, were injected. Dissection following the end of the experiment yielded a thyroid gland three times the size of normal. Histology under the microscope showed characteristics (Fig. 3) similar to those observed by Eitel & Loeser /12/ on thyroid glands of guinea pigs which had changed greatly after the administration of hypophysis frontal lobe powder. Against expectation, the body weight of this rabbit also increased, in spite of the inhibiting effect of thiouracil and tocopherol with respect to the normal thyroid function. This fact is relevant proof of the appreciable growth and development enhancing effect of Vit. E, which has also been found in tadpoles /12/.

The histological SD characteristics of rabbits treated with tocopherol always showed gland activities of a magnitude dependent on dosage. Very high epithelia and small follicles containing little colloid are characteristic of thyroids activated by tocopherol. The effect of Vit. E on the cell behavior

of the thyroid gland is clearly seen in Fig. 1 and 2. The question whether a direct relationship exists between tocopherol and the endocrine gland will be investigated later. Only a single indication exists at this yime that Vit. E in the form of wheat germ oil stimulates the normal growth of tissue in heart cultures /14/.

The differing effect of tocopherol on young rabbits of both sexes, especially with respect to the thyroid gland, supports the following view concerning the action mechanism: under the effect of alpha-tocopherol given per os, the hypophysis is stimulated to increased production of gonadotropic and thyreotropic hormones in the entire body. In the male body, however, the latter hypophyseal hormone is secreted in larger amounts than in the female body. This conclusion is reached by the following observations: (1) SD gain in weight by male animals, also observed by Hertz & Roberts /15/ in rabbits during their entire treatment with thyreotropic hormone, and (2) the normal iodine content in the thyreoidea of female animals and the iodine deficiency in the thyroid of male animals.

As is known, close relationships exist between thyroxin and thyreotropic hormones; the increased production of a thyreotropically effective hypophysis product thus leads reduced thyroxin formation or its complete cessation. The result is epthelial hyperplasia. This control of thyroid activity by the thyreotropic hormone occurs by humoral action. SD damage by a non-physiological increase in the E factor dose is the result of equilibrium distrubances of the endocrine gland system.

In addition, the following appreciable organic changes were observed during short and long-term feeding experiments: tocopherol-fed rabbits matured sexually earlier than normal animals of the same age. Their ovaries and testicles doubled in size during the Vit. E treatment. These observations confirmed the results of Stahler & al. /16/.

Similar pathological SD changes with respect to increased epithelia formation were also found in earlier rabbit experiments with wheat feeding /18/. Pathological phenomenn, as described above, were observed in the developing period of children /17/. It should be possible therefore that the Vit. E content of wheat flour is the cause of the histological organic damage reported above. It may be assumed that even small tocopherol doses lead to SD disturbance in humans, following longterm administration, especially with a monotonous diet.

According to one report /19/, the Vit. E content of 100 g wheat flour (Type 1050;mill degree 83%) amounts to 6-7.5 mg; this amount of flour approximately equals two slices of white bread or two hard rolls. The daily Vit. E requirement of a person is estimated to be 10-25 mg. In another paper /20/, the daily requirement of an adult is given as 1 mg, which in the authors opinion is inadequate.

It should be noted that presumably the danger of an excessive tocopherol supply is present even if the flour has been bleached with NCl_3 and benzoyl peroxide and the active substance thus partially destroyed /21/. Since the vitamin E content of wheat and .... /illegible/ flour is nearly equal, it is the author's opinion that not the vitamin content but a trace element, possible fluorine, is the cause of the different .... /illegible/ effectiveness of the two types of flour /18/.

It is intended to determine in subsequent experiments whether it is necessary to add the halogen to wheat flour to improve the quality of the latter. Investigations on animals in connection with their nutrition with respect to the effect of Vit. E on the organism will be continued, specifically to determine whether ascorbic acid activates or inhibits the action of tocopherol.

The author expresses his appreciation for the assistance by Prof. A. Dietrich, Stuttgart, in the evaluation of thyroid gland preparations and thanks Miss E. Schreiber for her skilled cooperation in the conduct of the experiments.

TABLE. Experimental Results with Rabbits Treated with Vitamin E. Daily Ration: Roots, hay and 10 g oats mixed with different amounts of tocopherol; Rabbits 483 and 484 received during the last 58 experimental days, instead of roots, 1 kg/day white cabbage, cooked, pressed and rinsed with distilled water. The low values are the result of glycogen determination with alcohol denatured with pyridine.

Key: 1. Number, sex, breed of animal; 2. Amount of substance and experimental period; 3. Weight in g; 4. Initial; 5. Increment; 6. Terminal; 7. Appearance 8. Liver; 9. Fresh weight in g; 10. % of body weight; 11. % glycogen; 12. mg% iodine; 13. Histology; 14. Thyroid gland; 15. Small, empty follicles, occasionally alveolar cells; high epithelium exhibiting bubble-shaped nuclei; strong SD hyperplasia and colloid secretion; 16. Enlarged follicles with high epithelium; mainly free of colloid, partially with finely crumbling content; hyperplastic SD with secretion; 17. High follicle epithelium with pale red protoplasma and large round nuclei. Irregular clearings without colloid (Fig. 1); 18. Glandular small blisters and fine alveolar cells with high epithelium, mostly without colloid; the lumina in part contain granular matter; hyperplastic thyreoida; 19. Large follicles, most or one-half of colloid; cubic cells with dcl. thick nuclei; lumina show alternating granulation and precipitated colloid; 20. Large colloid-containing follicles with flat epithelium and flattened nuclei. Small empty blisters with high epithelia, non-sperical cell nuclei; pale and red colloid contents. Transition present; 21. Broad follicles, large cells, light-colored protoplasma, finely grained; Colloid uniformly pale red to red. Large and whole epithelium (Fig. 2); 22. Follicle with slight uniformity, with colloid. Large cells with finely grained protoplasma, partial colloid precipitation; hyperplasis with partial flooding; 23. Partially colloid-containing cells; mainly flooding; hyperplasis, otherwise as in the section of Rabbit 483; 24. Irregular follicles; finely

Tier-Nr. Geschlecht Rasse (1)	Substanzmenge und Versuchsdauer (2)	Gewicht in g (3)			Leber (8)				Schilddrüse (14)				Histologischer Befund (13)
		Anfangs- (4)	Zunahme (5)	End- (6)	Aussehen (7)	Frischgew. in g (9)	% des Körpergew. (10)	% Glykogen (11)	Aussehen (7)	Frischgew. in g (9)	mg/kg Körpergew. (11)	mg% Jod (12)	
(25) 4[illegible] ♂ Dunkelblauer Wiener	0,52 g Vitamin E 13. 2. — 11. 3. 1947 26 Tage (35)	2550	+ 110	2660	gelbbraun 36	89,2	3,55	8,82	blaßrot (43)	0,17	64	0	Kleine, leere Follikel, mitunter wabige Zellen; hohes Epithel, das bläschenförmige Kerne darbietet; starke SD-Hyperplasie und Kolloidausschwemmung. (15)
(26) 472 ♂ Deutscher Scheck	0,94 g Vitamin E 19. 7. — 4. 9. 1946 47 Tage	1810	+ 100	1910	blaß 37	61,1	3,19	4,55	rot (44)	0,14	73	4,5	Vergrößerte Follikel mit hohem Epithel, überwiegend kolloidfrei, mit teilweise feinkrümeligem Inhalt. Hyperplastische SD mit Ausschwemmung. (16)
(27) 470 ♂ Blauweißer Wiener	0,8[illegible] g Vitamin E 13. 2. — 13. 5. 1946 89 Tage	1830	+ 870	2700	schokoladenbraun 38	85,5	3,17	1,36	blaßrot (45)	0,61	226	0	Hohes Follikelepithel mit blaßrötlichem Protoplasma und großen runden Kernen. Unregelmäßige Lichtungen, vorwiegend ohne Kolloid (Abb. 1). (17)
(28) 4[illegible] ♂ Französisch. Silber	1,66 g Vitamin E 14. 3. — 10. 6. 1947 88 Tage	1620	+ 1070	2690	gefleckt 39	93,8	3,48	12,14	blaßrot	0,23	85,5	4,92	Drüsenbläschen und feinwabige Zellen mit hohem Epithel, meist ohne Kolloid; z. Tl. enthalten die Lumina Körnelungen; hyperplastische Thyreoidea. (18)
(29) 476 ♀ Holländer	0,94 g Vitamin E 19. 7. — 4. 9. 1946 47 Tage	1700	+ 440	2140	braun 40	59,6	2,79	3,5	blaßrot	0,13	61	11,49	Gr. Follikel, meist b. z. Hälfte kolloidfrei. Kub. Zellen m. dkl., dick. Kernen; Lumina zeigen abwechs. Körnelung u. gefülltes Kolloid. (19)
(30) 487 ♀ Silbergrauer Scheck	1,96 g Vitamin E 13. 12. 46 — 11. 3. 47 95 Tage	1750	+ 720	2470	braun 40	86	3,48	8,34	blaß (46)	0,09	37	18,47	Gr. kolloidhalt. Follikel m. flach. Epithel u. abgeplatt. Kernen. Kl. leere Bläschen m. hoh. Epithel u. kugelig. Zellkernen; blaße u. rote Kolloidinhalte. Übergäng. vorhd. (20)
(31) 474 ♀ Rheinischer Scheck	0,95 g Vitamin E 19. 7. — 22. 10. 1946 95 Tage	1800	+ 680	2480	braun 40	65,9	2,66	3,14	blaßrot	0,12	43,4	11,1	Breite Follikel, große Zellen, helles Protoplasma; feinkörnig. Kolloid, gleichmäßig rot bis blaßrot. Epithel groß und hell (Abb. 2). (21)
(32) 48[illegible] ♀ Schwarzer Riese	1,34 g Vitamin E 1. 11. 46 — 7. 1. 47 67 Tage	2200	+ 200	2400	schokoladenbraun 39	45,1	1,88	0,46*	blaß sattrote Stellen (47)	0,12	50	14,55	Wenig gleichmäßige Follikel mit Kolloid; große Zellen mit feinkörnigem Protoplasma, z. Tl. Kolloidfällung; Hyperplasie m. mäßiger Ausschwemmung. (22)
(33) 4[illegible] ♀ Schwarzloh	1,34 g Vitamin E 1. 11. 46 — 7. 1. 47 67 Tage	2700	— 50	2650	dunkelbraun 41	43,7	1,64	0,95*	blaßblaßrot (48)	0,07	26,4	8,75	Teilweise kolloidhaltige Zellen; überwiegend Ausschwemmung; Hyperplasie; sonst wie Schnitt Kan. 483. (23)
(34) 492 ♀ Grausilber	0,44 g Vitamin E + 0,935 Thiouracil 27. 2. — 21. 3. 1947 22 Tage	2100	+ 290	2390	schokoladenbraun 42	79	3,3	3,97	sattrot (49)	0,43	180	0	Unregelmäßige Follikel; feinwabige Zellen, nur Kolloidreste; starke Blutfüllung; erheblich aktive Schilddrüse (Abb. 3). (24)

Tabelle. Versuchsergebnisse bei den mit Vitamin E behandelten Kaninchen.

Tägliches Futter: Dickwurz, Heu und 10 g Hafer, der mit verschiedenen Tokopherolmengen versetzt war; Kan. 483 und 484 bekamen in den letzten 58 Versuchstagen statt Dickwurz pro die 1 kg Weißkraut, das gargekocht, abgepreßt und mit dest. H_2O nachgespült war.

Die niedrigen Werte sind auf die Ausführung der Glykogenbestimmung mit pyridinvergälltem Alkohol zurückzuführen.

alveloar cells, colloid residues only, strong blood precipitation, considerably active thyroid gland (Fig. 3); 25. Darl blue Viennese; 26. German dappled; 27. Blue-white Viennese; 28. French silver; 29. Deuth; 30. Silvery gray dapple; 31. Rhenish dapple; 32. Black giant; 33. Black; 34. Gray silver; 35. Days; 36. Yellowish-brown; 37. Pale; 38. Chocolate brown; 39. Spotted; 40. Brown; 41. Dark brown; 42. Chocolate brown; 43. Pale red; 44. Red; 45. Pale red; 46. Pale; 47. Pale deep red spots; 48. Pale-pale red; 49. Deep red.

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Electron Microscopic Observations on the Anterior Pituitary of the Pregnant Mouse Administered with Vitamin E

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Introduction

Since Rinehart and Farquhar (1953) reported the cytology of the anterior pituitary of normal rat by electron microscopic observations, the extensive research has been carried out on the subject by many investigators (Rinehart and Farquhar 1953; Farquhar and Rinehart 1954 a, b; Green and van Bremen 1955; Yamada and Sano 1961; Lever and Peterson 1960; Barnes 1961; Hymer et al. 1961; Barnes 1962; Yamada 1963; Barnes 1963; Young et al. 1963; Siperstein et al. 1965). Among these observations, Barnes (1963) reviewed the cellular origins of five of six known adenohypophyseal hormones, namely they are GH, LTH, FSH, LH and TSH, in the mouse by electron microscopic studies of the responseness of anterior pituitary cells to estrus cycle, castration and treatment of thyroid hormone.

On the other hand, reproductive disturbances of rats associated with vitamin E-deficiency were first reported by Evans and Bishop (1922), subsequently the fetal absorption induced by maternal vitamin E-deficiency after 4~5 months of deficient diet was described by several authors (Masaoka 1932; Barrie 1937; Yonekawa 1938; Noda 1942; Horikawa 1958; Sasaki 1960) and moreover Barrie (1937) and Sasaki (1961) found that administration of vitamin E to the pregnant vitamin E-deficient rats produced the normal young.

Furthermore, recently in the literatures in which the relationship between vitamin-deficiency and pregnancy of animals was studied, it was reported that ovarial hormones diminished the disturbances of vitamin-deficient pregnant animals. Hays and Kendall (1965)

showed that the administration of progesterone exhibited the beneficial effect on the fetal growth in vitamin A-deficient rabbit. Cheng (1959) noted that in maternal vitamin E-deficiency the incidence of congenital malformations were reduced by the treatment of both estrogen and progesterone and Nelson et al. (1953) stated that the administration of both estrogen and progesterone maintained the gestation in pyridoxine-deficient rat.

On the point of close relationship between vitamin and ovarial functions or vitamin and functions of pituitary gonadal system as suggested by literatures described above, the author presents this experiments to clarify the function of vitamin E. In experiments reported here by the author vitamin E was examined in pregnant mice and ultrastructural changes of their anterior pituitaries were observed with electron microscope.

Materials and Methods

A total of 35 mature female mice of dd strain were used in this experiments. They were divided into three groups. The first group were 5 intact mice in proestrus stage. The second group were 15 mice during late pregnancy. of which the first 5 mice were the 11th day of gestation. the second 5 mice were the 14th day of gestation and the third 5 mice were the 18th day of gestation. Pregnancy in the mice was confirmed by the presence of sperm in vaginal smear after mating with normal male. The third group were the same of the second group, however they were injected daily with 10 mg of vitamin E from the 1st day of gestation. Each animals was killed by decapitation. The pituitary gland was dissected out quickly and placed in the cold buffered isotonic OsO_4 dropped on the wax plate, then the anterior pituitary was cut into blocks less than 1-2 mm on a side and fixed with another buffered isotonic 1% OsO_4 at 4 C for 1 hour. After fixation. the blocks were washed in the Ringer's solution. rapidly dehydrated in ethanol series and then embedded in Epon 812 according to the Luft's method. The thin sections were obtained with JUM 4 ultramicrotome (Japan Electron Optics Laboratory Co., Ltd.). and mounted on copper grids. The sections were stained in a 0.5% uranyl acetate alcohol solution for 1 hour and then washed in distilled water. Sections were observed with HUA 11 electron microscope (Hitachi Ltd. Tokyo Japan).

Observations

Normal female mice

1) GH cell:

These cells (Fig. 1) are the most frequently encountered type cell in the anterior pituitary, rounded or oval in shape, and easily recognized by the presence of secretory granules which are relatively large in number and possess the electron dense material and rounded in outline. The maximum diameter of the secretory granules are approximately 350 mμ in general, but occasionally 400 mμ. These granules are scattered randomly throughout the cytoplasm in some cells, and in still others they are concentrated in the one part of cell periphery, but as a rule they are not observed in perinuclear areas or within the cysternae of the rough surfaced endoplasmic reticulum. Secretory granules observed in the Golgi region are enclosed clearly by a thin membrane, relatively lower electron dense and smaller in size than those in the cell periphery.

The mitochondria are relatively larger. their outlines are rod shaped and they possess the moderately electron dense material and occasionally their outer surfaces are found closed to the exterior of rough surfaced endoplasmic reticulum.

Commonly rough surfaced endoplasmic reticulum as a series of flattened sacks in parallel rows is arranged in the perinuclear area or is stacked to the cell border, but sometimes its cysternae are partially dilated.

In the perinuclear area. condensed Golgi apparatus is observed and it contains a reduced number of vacuoles. vesicles and fine lamellae. Occasionally a few number of vesicles contain the low electron dense material.

2) LTH cell:

These cells (Fig. 2) are moderate in size and usually oval or irregularly rectangular in outline. They are easily identified by the presence of oval or irregularly shaped secretory granules ranging from 150 mμ to 400 mμ in their longest dimension. The secretory granules are scattered moderately throughout the cytoplasm except region occupied by rough surfaced endoplasmic reticulum. Among the secretory granules encountered in the Golgi region. a few number of them occasionally contain the partially different electron dense particles within them.

The mitochondria are tiny rod-like in shape and smaller in

size than those of GH cells.

In general rough surfaced endoplasmic reticulum is arranged in flattened parallel lamellae at periphery of the cytoplasm, occasionally it is in the form of small vacuoles. Rough surfaced endoplasmic reticulum occurred as a small vacuole in the Golgi region is studded with poor ribosomes partially and it resembles the small Golgi vacuole.

The Golgi apparatus develops moderately and occupies the area of cytoplasm between the nucleus and rough surfaced endoplasmic reticulum observed in the form of flattened lamellae at the one part of cell periphery. The small secretory granules consisted of electron dense materials are observed within the Golgi vacuole.

3) FSH cell:

These cells (Fig. 3) are large in size, oval or irregularly rectangular in shape and are adjacent to blood vessels. The spherical secretory granules ranging from 150 mμ to 200 mμ randomly scattered throughout the cytoplasm and they are enveloped by a thin membrane and are lower electron dense than those of other type cells, but the center of the secretory granules are somewhat electron dense than the periphery of granules.

The mitochondria are tiny rod-like in shape or occasionally Y shaped and their matrices are relatively electron dense. Rough surfaced endoplasmic reticulum of these cells is scattered sparsely in the cytoplasm, and shows mainly irregularly shaped sacks but sometimes flattened sacks.

The nuclei are occasionally hollow and the voluminous ratio of cytoplasm to nucleus is larger than in other type cells. In general the Golgi apparatus is poorly developed and small in size.

1) LH cell (Fig. 1)

They are relatively small and round, oval and irregularly rectangular in shape. They are adjacent to blood vessels like FSH cells.

The spherical secretory granules ranging from 160 mμ to 200 mμ in size are more electron dense than those in FSH cells, and mainly observed at the cell periphery.

In general mitochondria are tiny rod-like in shape.

Rough surfaced endoplasmic reticulum is moderately developed and in some cells it occurs as a series of flattened sacks in a few layers around the nuclei or it is stacked to the one part of cell periphery and occasionally randomly scattered as irregularly shaped sacks throughout the cytoplasm.

The Golgi region is relatively small and contains a few number of small vacuoles, several vesicles and fine lamellae.

5) TSH cell (Fig. 5)

In general they are polygonal in shape.

The spherical secretory granules are small and range from 50 mμ to 100 mμ in size. Characteristically they are often arranged in a row at the vicinity of the cell membrane.

Rough surfaced endoplasmic reticulum is poorly developed and occasionally appears as flattened sacks sparsely scattered in the cytoplasm.

Generally the Golgi apparatus is reduced in size and developed poorly.

6) Agranular cell:

This type cell is divided into two groups as described below.

a) The first group (Fig. 6):

Generally these cells are small and observed in small groups but rarely encountered. They are oval or polygonal in shape and show relatively abundant desmosomes at the cellular margin. Their cytoplasm are relatively electron lucent.

The mitochondria are tiny rod-like in shape and poor in number and moreover their cristae are relatively small in number.

Rough surfaced endoplasmic reticulum is poorly developed and appears as a few vesicles or flattened sacks and their outer surfaces are studded partly with ribosomes.

In the vicinity of nuclei the reduced Golgi region is observed.

b) The second group (Fig. 7):

The cells of this group are relatively large in size and oval in shape.

The mitochondria are rod or Y shaped in outline. Rough surfaced endoplasmic reticulum is sparsely scattered in the form of small round or flattened sacks throughout the electron lucent cytoplasm. The Golgi apparatus is generally small in size and shows fine membrane systems. Besides vesicle-like bodies with electron transparent material are also scattered in the cytoplasm.

Pregnant mice

1) GH cell:

These cells show an increase in size during the second half of gestation, in particular after the 14th day of gestation as compared with normal female mice. They are mainly oval in shape

and well granulated (Fig. 8, 9, 10).
Mitochondria are sometimes observed as swollen, relatively electron lucent rods and their cristae are arranged irregularly.
They are not observed among rough surfaced endoplasmic reticulum. Rough surfaced endoplasmic reticulum shows compact lamellae of flattened sacks or irregular arrangement (Fig. 9, 10) and is stacked to the one part of cell periphery.
The Golgi region is in general hypertrophied and occupies the cytoplasmic area fronted on the hollowed outline of nucleus (Fig. 8, 9). In the Golgi region several vacuoles and many vesicles filled with electron dense material are observed (Fig. 9) and moreover immature secretory granules clearly enveloped by a thin membrane are recognized too.

2) LTH cell:
In comparison with normal female mice these cells are enlarged and exhibit the hypertrophy of cytoplasmic membrane systems during late pregnancy, particularly after the 14th day of gestation (Fig. 11).
Their secretory granules are somewhat decreased as compared with normal female mice (Fig. 11, 12).
Well developed rough surfaced endoplasmic reticulum is observed as compactly parallel lamellae which is situated parallel to the outline of the nuclei and is stacked to the one part of cell periphery or distributed in the whole cytoplasm except the Golgi region.
The Golgi area (Fig. 11) is enlarged in size as compared with normal female mice and it indicates a several number of vacuoles and many vesicles filled with electron dense material.
After the 14th day of gestation, some LTH cells appear characteristically and their secretory granules are very reduced in number while the cytoplasmic membrane systems are greatly hypertrophied (Fig. 12). These characteristic LTH cells increase gradually in number following the process of gestation.

3) FSH cell:
During the second half of pregnancy, these cells are somewhat enlarged in size.
The secretory granules are increased particularly after the 14th day of gestation in comparison with normal female mice (Fig. 13).
Like normal female mice rough surfaced endoplasmic reticulum shows only a slight development is scattered in the cytoplasm as irregularly shaped sacks or partly flattened sacks.

4) LH cell:
These cells are increased in size during pregnancy, especially enlarged markedly during the second half of pregnancy. They are oval or irregularly rectangular in shape.
Their secretory granules generally observed in the cell periphery are somewhat increased as compared with the normal female mice.
Rough surfaced endoplasmic reticulum is prominent and it is observed as irregularly shaped sacks or partly dilated tubules throughout the cytoplasm.
The Golgi region is well hypertrophied in the vicinity of the nucleus as compared to normal female mice (Fig. 15, 16) and they are arranged like a horseshoe. The Golgi apparatus consists of several vacuoles, many vesicles and moderately developed lamellae. Within the Golgi vesicles materials of different electron density are observed.

5) TSH cell:
These cells exhibit no ultrastructural changes in comparison with normal female mice except a slight increase of rough surfaced endoplasmic reticulum which shows flattened sacks.

6) Agranular cell:
a) There are no ultrastructural changes between these cells of pregnancy and those of normal female mice.
b) Ultrastructural differences are not observed between these cells of pregnancy and those of normal female mice except a slight increase of these cells in pregnancy.

Pregnant mice treated with vitamin E

1) GH cell:
In these experiments they become larger in size as the gestation period proceeds, particularly after the 14th day of gestation they are markedly enlarged. These cells are mainly oval in shape and majority of them are heavily granulated (Fig. 17).
The secretory granules are generally scattered throughout the cytoplasm except the Golgi region. In the periphery of the cell, progressive lowering of electron density of secretory granules, of which limitting membranes are adhesive with slightly hollowed cell membrane, are observed.
The mitochondria are sometimes swollen and contain electron lucent matrix and their cristae show irregular arrangement.
In comparison with normal female mice, rough surfaced endoplasmic reticulum is well developed as parallel lamellae or irregular form, that is, the section of cysternae communicated each

other, and occupies the greater part of cytoplasm except the Golgi region (Fig. 19).

The Golgi region (Fig. 18) is well enlarged and the Golgi apparatus consists of relatively numerous vacuoles, abundant vesicles and partly dilated lamellae. A few vacuoles include partially electron dense material and many vesicles contain low electron dense material. Some secretory granules recognized in the Golgi region are enveloped clearly by a thin membrane and occasionally their electron density is somewhat lower in comparison with mature secretory granules.

A few number of lysosomes which are irregular or oval in shape are observed. In size they are about 1μ in maximum and contain generally electron dense material, but sometimes partly electron lucent material.

2) LTH cell:

During pregnancy these cells are enlarged, in particular more markedly enlarged after the 14th day of gestation than normal female mouse. They are mainly oval in shape. The secretory granules are decreased in number after the 14th day of gestation and their longest dimension becomes somewhat shorter (Fig. 20).

Conspicuous development of rough surfaced endoplasmic reticulum occurred as parallel arrays is observed (Fig. 20) in the greater part of cytoplasm except the Golgi region, and its maximal development is found after two weeks of gestation.

The mitochondria are sometimes devoid of cristae partly and contain electron lucent matrix. A few number of them touched to the outer surface of the Golgi vacuole or rough surfaced endoplasmic reticulum.

As compared with normal female mice, the Golgi region is markedly hypertrophied after two weeks of gestation and it consists of many vacuoles and abundant vesicles, some of which contain electron dense material. A few number of rough surfaced endoplasmic reticulum are recognized in the Golgi region and the parts of their exteriors fronted to the Golgi vacuole are not studded with ribosomes.

Occasionally a few lysosomes appear in cytoplasm and they are moderate in size and oval in shape.

3) [illegible] cell:

In these experiments these cells are enlarged during the second [illegible] of gestation and they are oval or roundly polygonal in shape.

After the 14th day of gestation the secretory granules are markedly increased and they occupy the greater part of cytoplasm (Fig. 21).

Mitochondria exhibit no morphological changes during the all pregnancy period.

Rough surfaced endoplasmic reticulum is not developed but sometimes it is observed mainly as flattened sacks.

The Golgi apparatus is poorly developed (Fig. 21).

In the cytoplasm appear a few lysosomes which are irregular or oval in shape. The irregular lysosome contains electron dense material and the oval one contains slight electron lucent material in which especially at its periphery small amount of electron dense material is observed. Both of them are enveloped by a thin membrane.

4) LH cell:

They are oval or polygonal. As the gestation proceeds, they are increased in size, especially markedly increased after two weeks of gestation (Fig. 23).

The secretory granules are observed mainly at the cell periphery and they are somewhat reduced in number after the 14th day of gestation in comparison with normal female mice.

Rough surfaced endoplasmic reticulum is prominent as compared with that of normal female mice and it is scattered throughout the cytoplasm and exhibits irregularly shaped sacks or partially dilated tubules.

Some mitochondria possess the irregularly arranged cristae and electron lucent matrix. Occasionally they touched to the exterior of rough surfaced endoplasmic reticulum.

Differing from normal female mice, the Golgi apparatus arranged in a horseshoe is well hypertrophied in the vicinity of nucleus (Fig. 22, 23) and it consists of many vacuoles and abundant vesicles and moderately developed lamellae (Fig. 24). Among the Golgi vacuoles a few number of them are partly filled with electron dense material.

A few lysosomes appear in the cytoplasm and they are moderate in size.

5) TSH cell:

In comparison with normal female mice morphological changes are not observed except slight development of rough surfaced endoplasmic reticulum which shows sometimes parallel arrays of a few flattened sacks in the cytoplasm.

Occasionally the Golgi apparatus is recognized.

6) Agranular cell:
   a) No ultrastructural changes are observed in these cells in comparison with normal female mice.
   b) The ultrastructural differences are not observed as compared with normal female mice except a slight increase of rough surfaced endoplasmic reticulum recognized as flattened sacks.

Discussion

Microsome obtained from tissue homogenate by the technique of centrifugation has exhibited membranes studded with small particles in electronmicroscopy (Palade & Siekevitz 1956; Kuff et al. 1956; Hanzon & Toschi 1959). Therefore microsome is morphologically identical with rough surfaced endoplasmic reticulum. Studies of Barnum & Huseby and Takanami reported the heterogeneity of these submicroscopic components of cytoplasm, that is, one component is rich in lipid, the other component is rich in RNA. On the other hand, partial or total disappearance of small particles of microsome was recognized in the incubation of microsome with ribonuclease by Palade & Siekevitz and so they suggested that RNA is localized in the small particles.

Moreover, Littlefield et al. (1955) prepared ribonucleoprotein particles which contain equal amount of RNA and protein, from the microsomal fraction of rat liver by the use of desoxycholate and hence they evidenced that the small particles consist of ribonucleoprotein and the membranes containing mainly lipid.

Since the relationship between RNA and protein synthesis was emphasized by Casperson (1941) and Brachet (1943), a number of investigations on the important role of nucleoprotein complex of microsomal fraction in protein synthesis were reported.

By Littlefield et al. (1957) the incorporation of intravenously injected radioactive amino acid into the protein of small particles was proved and moreover Arakawa (1960) observed that after dl-ethionine administration ribosomes disappeared in several portions of rough surfaced endoplasmic reticulum in hepatic cells of albino rats.

From a number of studies described above, the important role of rough surfaced endoplasmic reticulum in protein synthesis is easily understandable, however by Watanabe (1956) it was stated that rough surfaced endoplasmic reticulum was very poor and abundant free ribosomes appeared in myeloblasts which were usually in rapid

growth. Therefore we may understand from these reports that rough surfaced endoplasmic reticulum has the important role in protein synthesis involved in specific function of the cell, for example the secretion of proteinaceous hormone.

In fact, recent study of Caro & Palade (1964) by electron microscopical autoradiography in the pancreatic exocrine cell using dl-leucine-4.5 H^3 as label has shown that the label is early associated with rough surfaced endoplasmic reticulum and later it appears in zymogen granules.

So the development of rough surfaced endoplasmic reticulum in the proteinaceous hormone-secreting cell probably means the active function of secretion.

Because the Golgi complex did not appear in any recognizable form among the various fractions obtained by differential centrifugation, its component and biochemical function are not clarified.

However the important role of the Golgi complex in secreting mechanism was shown by recent studies of Caro & Palade and Peterson & Leblond (1964). By electron microscopical autoradiography using dl lucine-4.5 H^3 as label Caro & Palade in their studies of pancreatic exocrine cell have shown that the label appears early in region occupied by rough surfaced endoplasmic reticulum, next in the Golgi complex and later in zymogen granules. Moreover by the use of d glucose-6-H^3 as label Peterson and Leblond have suggested that an important role of the Golgi complex is to provide for the synthesis of glycoprotein.

From these studies an important role of the Golgi complex in secreting mechanism is easily understandable. Therefore the development of the Golgi complex in the hormone-secreting cell may exhibit a sign of hyperfunction of secretion.

The physiological function of vitamin E has been investigated mainly with respect to its deficiency in animals since discovery of essential function to reproduction by Evans and Bishop (1922).

Since many years the necessity of vitamin E for proper function of the anterior pituitary has been a controversial problem. Barrie stated that in the anterior pituitary of vitamin E deficient rat the acidophiles were very small and degenerated, and then also some of the basophiles were degenerated. Moreover it was found that stillbirths were relatively high and the growth of litters was abnormally slow. Müller and Müller (1937) kept female rats on a vitamin E-free diet for 137~240 days with no morphological changes in the anterior pituitary. Biddulph and Meyer (1941) found that in

rats fed on a diet deficient in vitamin E the weight of female anterior pituitary was not different from that of the normals and that pituitaries from females showed the same ovarian response as those from normal females.

Herrick et al. (1952) observed that the anterior pituitary from the birds on the E-deficient diet had approximately one half as many active basophile cells as those from the bird with the vitamin E supplied. At the same time they found in the pituitary glands of vitamin E-deficient male chickens less gonadotropic hormone than those in vitamin E supplied male chickens. Recently Lee (1960) and Ho (1961) have emphasized that by a deficiency of vitamin E, a number of both acidophiles and basophiles are primarily degenerated in the anterior pituitary of rats and that the residual chromophiles are hypertrophied and their cytoplasmic stainability becomes intense. Furthermore Sasaki (1960b) showed that by the administration of vitamin E both of pituitaries and ovaries from mature female rats increased in weight. As described above, studies concerning the influence of vitamin E-deficiency upon the function of the anterior pituitary are conflicting.

However Beckmann (1955) found that in the pituitary the level of vitamin E concentration was most highest of all organ, consequently it is suggested that vitamin E acts primarily on the function of the anterior pituitary.

Since the discovery of antioxidant activity of vitamin E by Olcott and Mattill (1941), a number of investigations have been made on vitamin E as a lipid antioxidant. Dam (1957) has summarized the strong evidence that vitamin E functions entirely as a lipid antioxidant in vivo.

Concerning the composition of microsome Littlefield et al. (1955) proved that the membranes contained mainly lipid, and moreover Tappel (1962) stated that microsome contained 30~40% total lipid, mainly phospholipid, had twice the amount of polyunsaturated lipid as that of mitochondria. These polyunsaturated lipid are very oxygen-labile and easily oxidized. Lipide peroxidation is a source of free radicals which is able to react at random in the microsomes and therefore should be damaged seriously to the microsomal enzymes.

In fact, recent studies of the damage to cytochrome c by linoleate peroxidation of Desai (1962) showed that cytochrome c catalyzed the peroxidation of linolenic acid and in turn free radicals produced by the catalyzation attacked cytochrome c, then cytochrome c lost its solubility. Because the vitamin E acts as a lipid antioxidant, enzyme-

inactivation of microsome concurrent with lipid peroxidation does not occur by added vitamin E.

On the other hand, because lipid acts as a cement substance of electron transport sequence in mitochondria, as studied by Green & Crane (1958) it is suggested that also in membranes of microsome lipid acts as a cement substance of electron transport sequence. Therefore lipid peroxidation has profound effect on enzymes in membranes.

In the condition of hyperfunction of the secreting cell, much energy is required for the maintenance of secreting mechanism. This energy source is the high energy phosphate bond, that is ATP, which is produced mainly in mitochondria by oxidative phosphorylation. In the microsome, DPNH cytochrome reductase, TPN cytochrome reductase, flavoprotein and cytochrome b_5 were recognized by many workers.

Consequently it is assumed that also in microsome oxidative phosphorylation is accelerated as those in the mitochondria when abundant energy is required for the maintenance of secreting mechanism, in other word, high level of oxidation is present and so the elevated requirement of vitamin E which is the only known lipid antioxidant in microsome is necessary for the protection of lipid from oxidation in the state of hyperfunction of secretion.

In fact Tappel observed that by electron microscopy the microsome of vitamin E deficient animals was degenerated.

Finally, the author should discuss that interrelationship between vitamin E and electron microscopical observations of anterior pituitary.

GH cell:

During gestation, in particular after the 14th day of gestation, the animals showed marked changes of this type cell in comparison with the normal.

Rough surfaced endoplasmic reticulum was developed and the Golgi region was hypertrophied. In the Golgi region, granules being formed within the Golgi elements were observed.

From these changes it is readily understood that the secretory function is elevated. In such conditions polyunsaturated lipid of rough surfaced endoplasmic membranes is easily oxidized and therefore the administration of vitamin E which is the only known lipid antioxidant in microsome is significant, that is, vitamin E maintains the function of rough surfaced endoplasmic reticulum by the protection of their polyunsaturated lipid from oxidation. In fact, in this report the animals which received vitamin E showed readily recognizable changes as compared with pregnant mice. Frequently abundant

secretory granules, well developed rough surfaced endoplasmic reticulum, greatly hypertrophied Golgi region, relatively many granules being formed within the Golgi elements and figures considered as granules being released are observed.

Thus, these changes may mean that GH cells have the secretory hyperfunction. Several investigations in which necessity of vitamin E for growth of rat was described by Evans (1928), Mason (1929), Blumberg (1935), Emerson & Evans (1937), Nelson et al. (1940), Evans et al. (1938) and Sasaki (1960) support these changes of GH cells.

LTH cell:

During the second half of gestation, both of rough surfaced endoplasmic reticulum and the Golgi region were enlarged, however, secretory granules were somewhat decreased in comparison with mature normal female mice. On the other hand, Hurst & Turner (1942) reported that during gestation, in particular after the 10th day of gestation, LTH content of pituitary gland increased by 88 percent in comparison with mature normal female. Apparently decreasing of secretory granules is inconsistent with increasing of LTH content of pituitary gland reported by Hurst & Turner. This conflict may be because of that a rapid rate of hormonal protein synthesis is occuring when the well developed rough surfaced endoplasmic reticulum is present and consequently the releasing of hormonal product is rapidly accelerated.

During the second half of gestation both of rough surfaced endoplasmic reticulum and the Golgi region developed and hence the function of hormon-producing is elevated.

In such conditions administration of vitamin E may be significant as described in comment regarding GH cells. In fact, pregnant mice which were administered with vitamin E showed the conspicuous development of rough surfaced endoplasmic reticulum, well hypertrophied Golgi region and a few number of secretory granules. These changes may mean that LTH cells have the hyperfunction of hormone-producing. These changes were substantiated by the observations in which scanty lactation of vitamin E deficient rat was stated by Barrie and Sasaki (1960b).

After the 11th day of gestation, some LTH cells characterized by the coexistence of a few number of secretory granules and the well developed cytoplasmic membrane systems are observed, that is, the rough surfaced endoplasmic reticulum occurs as compactly parallel lamellae which occupies the cytoplasm except the Golgi region which

is hypertrophied in the vicinity of the nucleus.

These cells may correspond to peculiar chromophobe cells reported by Yamada (1960) and Kato (1956) which possess an eccentrically situated nucleus and juxta nuclear area surrounded by a cap-like rim which is ribonucleic acid in nature, at the cell periphery except at the side attached to the nucleus.

FSH cell:

In pregnant mice no marked changes of these cells were observed in comparison with normal female mice except an increase in cellular size and number of secretory granules, in particular both of them are recognized clearly during the second half of gestation.

In pregnant mice a rapid rate of FSH synthesis is not occurring because both of rough surfaced endoplasmic reticulum and the Golgi region are not developed in these cells. Therefore an increase of secretory granules is considered as a result of their storage.

These changes are substantiated by Zeiner's observations (1952) in which higher gonadotrophin was recognized in pituitaries of pregnant rats during the last week of gestation.

In pregnant mice treated with vitamin E, the secretory granules are markedly abundant in comparison with normal pregnant mice.

This observation may correspond to Nakamura's observation (1960) in which in pregnant mice administered with vitamin E, basophilic stainability of anterior pituitary cells becomes intense as compared with normal pregnant mice.

LH cell:

In comparison with normal female mice these cells are hypertrophied and both of rough surfaced endoplasmic reticulum and the Golgi region are developed and hence hormone synthesis in these cells is considered to be elevated. In fact Zeiner stated that during the last week of gestation in rats gonadotrophin content was higher.

In pregnant female mice the administration of vitamin E is significant as described in comment regarding GH cells.

By the administration of vitamin E the cells are slightly increased in size and both of rough surfaced endoplasmic reticulum and the Golgi region are developed and the secretory granules are somewhat decreased in comparison with pregnant mice.

These changes are considered as the results of active secretory function. Moreover in view of observations by Evans, Masaoka, Yonekawa and Noda on vitamin E deficient rats, it was recognized that fetal absorption occurred during gestation and then if the deficient pregnant rat was dosed with vitamin E, the gestation

exhibited the normal procedure.

From these observations vitamin E is necessary for the normal function of the pituitary gland, in particular LH secretion during pregnancy.

However, on the other hand, the fact demonstrated Pencharz and Long (1933) that by hypophysectomy after 11th day of gestation pregnancy in the rat was not interrupted and moreover the fetal absorptions of vitamin E-deficient rat were observed by several investigations especially during later gestation and furthermore Sasaki reported that LH administration could not prevent fetal absorption in vitamin E-deficient rat.

These observations suggest that important role of vitamin E during later gestation is not only based on the pituitary functions, particularly function of LH secreting, but on functions of the placenta.

TSH cell:

Three groups, that is, normal female mice, normal pregnant mice and pregnant mice treated with vitamin E, exhibited no difference with one another in these cells.

From observations of Barrie, Lee and Ho that in vitamin E-deficient rat the thyroid was very hypoplastic, it is easily suggested that pregnant mice treated with vitamin E exhibit morphological changes of TSH cells in comparison with pregnant mice. However, in this experiment morphological differences of TSH cells are not observed among three groups.

Agranular cell:

a) Among three groups morphological differences are not recognized in these cells. In these cells both of rough surfaced endoplasmic reticulum and the Golgi region poorly developed, so it looks likely that a rapid hormone synthesis is not occuring in them.

b) Each experimental groups showed no morphological differences with one another in these cells.

One of these cells may correspond to corticotrophs described by Siperstein et al. (1965).

Moreover Heinsen and Körker (1951) reported that vitamin E was possible to activate directly the secretion of ACTH in pituitary gland and Lee and Ho stated that in vitamin E-deficient rats atrophy of adrenal cortex was observed.

From these studies it certainly looks likely that vitamin E has the direct effect on these cells, however in this experiment no morphological differences are observed.

Summary

Anterior pituitary from mature virgin female, pregnant and vitamin E-treated pregnant mice were studied with electron microscope. It was found that during gestation both of rough surfaced endoplasmic reticulum and the Golgi region developed well in GH cells, LTH cells and LH cells in comparison with normal mature female. Development of both intracellular organellae was in higher degree in anterior pituitaries of vitamin E-treated pregnant mice.

On the basis of these results, effects of vitamin E on anterior pituitaries of pregnant mice were presented with respect to secretion of GH, LTH and LH, that is, administration of vitamin E in pregnant mice activates the secretory function of GH, LTH and LH in anterior pituitary.

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The Fine Structure of Testicular Interstitial Cells in the Mouse Administered with Vitamin E

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Introduction

Since the reproductive disturbances of rats associated with vitamin E-deficiency were first reported by Evans & Bishop ('22), subsequently histological changes of testes induced by vitamin E-deficient diet in rats were described by many authors. In those reports, some authors noted that testicular degeneration induced by vitamin E-deficiency was found only in seminiferous tubules and not recognized in interstitial cells (Van Wagenen, '25; Mason, '26; Evans & Burr, '27; Kudrjaschov, '30, '31; Juhasz-Schaffer, '31, '32; Nelson, '33; Griesbach, et al. '57), while on the other hand several authors observed histological changes in interstitial cells of rats in such condition (Marchesi, '35; Ringsted, '36).

Furthermore, recently Fujiao & Yoshioka ('62) described that vitamin E administration activates the function of interstitial cells in mature mice. As described above, studies concerning the relationship between vitamin E and histological changes of interstitial cells of testes are not definite yet. In this study mature male mice were administered with vitamin E and the interstitial cells of their testes were investigated with electron microscope with respect to their ultrastructural changes. On the basis of this study, the author presents in this paper the close relationship between vitamin E and the function of interstitial cells of testes in mature male mice.

Materials and Methods

A total of 20 mature male mice of dd strain were used in this study. They were divided into two groups. The first group was 10

normal intact mice. The second group was 10 mice injected daily with 10 mg of vitamin E (dl-α-Tocopheryl acetate prepared by EISAI Co., Ltd.) for 20 days. Each animal was killed by decapitation. Bits of testes were dissected out quickly and fixed in buffered isotonic OsO_4 solution at 4°C for 2 hours. After fixation, the tissues were washed in Ringer's solution. rapidly dehydrated in ethanol series and then embedded in Epon 812 according to Luft's method. The thin sections were cut on a JUM-4 ultramicrotome (Japan Electron Optics Laboratory Co., Ltd.). and then stained in a 0.5% uranyl acetate alcohol solution for 1 hour. Sections were viewed in HUA 11 electron microscope (Hitachi Ltd. Tokyo Japan).

Observations

Normal Testicular Interstitial Cells (Fig. 1, 2, 3)

These cells are generally oval or irregularly rectangular in shape. Between adjacent cells many interstices (Fig. 1) are clearly observed. The cell surface exposed to the interstices is sometimes covered with microvilli and in some cases small hollowed cell surface studded with numerous microvilli is recognized at perivascular space (Fig. 3).

The mitochondria are moderate in size, commonly oval or rod-shaped and their internal matrices exhibit abundant irregular tubular cristae (Fig. 2).

In cytoplasm. smooth surfaced endoplasmic reticulum (sER) (Fig. 1) is abundant except in perinuclear area in which rough surfaced endoplasmic reticulum (rER) relatively well developed as small sacks or small flattened sacks. Mainly sER is generally recognized as vesicles. however occasionally it appeared as membrane whorls (Fig. 1) or cluster of vesicles which possess sometimes electron dense material in its center.

In area in which sER is abundant. a few number of double walled tubules (Fig. 2) are rarely observed. The outer membranes of this structure are occasionally continuous with neighbouring vesicular sER.

Osmiophilic dense bodies appeared in cytoplasm are divided morphologically into two types. Dense bodies of the first type are generally round in shape and bounded by a thin membrane. The second type of dense bodies shows irregular outline, and commonly appears in some areas of the cytoplasm in which sER is predominant and occasionally they are wrapped in several layers of flattened sacks of sER.

The Golgi apparatus recognized generally in perinuclear area is poorly developed. In the Golgi region, cytoplasmic inclusions corresponding to secretory granules are not present.

Testicular Interstitial Cells Treated with Vitamin E (Fig. 4, 5, 6, 7, 8)

In comparison with normal interstitial cells, they are hypertrophied and mainly oval in shape, and touched closely to adjacent cells.

As compared with normal testicular interstitial cells, marked development of sER (Fig. 5) is observed within them and it occupies the greater part of cytoplasm and especially sER observed as a membrane whorl (Fig. 6, 7, 8) or cluster of vesicles is increased in size and number. Between membrane whorls consisted of only membranous component (Fig. 7) and the structures with an electron dense core (Fig. 8), transitional figures are often observed.

The irregular osmiophilic dense bodies (Fig. 6) are slightly increased in size and number and occasionally their profiles contain many vesicles.

Concerning the Golgi apparatus, no differences are recognized between the normal and the treated.

Discussion

In interstitial cells of normal mice, sER is well developed as Christensen & Fawcett ('66) noted in their work and moreover this development of sER is recognized also in interstitial cells of other species by several authors (Christensen & Fawcett, '61; Porter & Bonneville, '64; Christensen, '65). Especially sER observed as membranous whorl, which was first reported by Carr & Carr ('62), is also found in the mouse.

The testicular interstitial cells are considered as the main source of steroid hormones produced in testes as clearly confirmed by histochemical studies (Baillie & Griffiths, '64; Maeir, '65). Furthermore by recent biochemical studies (Toren, et al., '64; Shikita, et al., '64; Lynn & Brown, '58; Horfmann, '62), testosterone is biosynthesized from cholesterol and the enzymes mediating the synthesis of testosterone from pregnenolone is contained in microsomal fraction and further enzymes involved in biosynthesis of cholesterol are present in microsomal fraction too (Ying, et al., '65), that is, sER of testicular interstitial cells is the principal site of these enzymes. Fawcett ('65) suggested that membranes of sER may play the role of reservoir of cholesterol.

In testes of mice treated with vitamin E, interstitial cells are hypertrophied and their sER is markedly developed in comparison with the normal. From studies described above, these changes may mean that the interstitial cells are hyperfunctional in steroid hormone production.

Concerning membrane whorls, they are more abundant in vitamin E administered mice in comparison with the normal and moreover transitional figures are often observed between the structure consisted of membranous component and that with electron dense material.

These figures may possibly be interpreted as the results of the requirements of much cholesterol and thereafter the releasing of own cholesterol of the sER in hyperfunctional state. These ultrastructural changes of testicular interstitial cells of the mouse administered with vtiamin E. which suggest an increased steroid hormone production, are well consistent with reports of Yamada & Ukei (57) and Ichihara (66) that administration of vitamin E activates the function of LH secretion of mouse anterior pituitary.

Summary

10 male mice of 14 weeks of age were treated daily with 10 mg vitamin E injection for 20 days. After the treatment, they were decapitated and their testes were examined by electron microscope.

1) By administration of vitamin E, testicular interstitial cells are enlarged and the sER is markedly developed, especially the membranous whorls are more abundant in comparison with normal interstitial cells.
2) From these data, it is conceivable that vitamin E administration activates the function of steroid hormone production in the testicular interstitial cells.

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Explanation of Figures

Fig. 1. Low power electron micrograph of a mouse interstitial cell (Isc) lying near seminiferous tubules (sem). In cytoplasm, sER is well developed as small vesicle and membrane whorl (arrow). ×3000

Fig. 2. Higher magnification of left upper part of Fig. 1. A several number of double walled tubules (dwt) are present. At the end of them, outer membranes are continuous with vesicle of sER. Mitochondria (Mt) are seen and their internal matrices exhibit abundant tubular cristae. ×10000

Fig. 3. Higher magnification of an interstitial cell. In middle upper part, the hollowed cell surface (arrow) is studded with numerous microvilli (mv). Mitochondria (Mt), sER and free ribosomes are seen. ×10000

Fig. 4. Testicular interstitial cells (Isc) of the mouse treated with vitamin E. These cells are rounded in shape and enlarged in comparison with the normal. The sER is well developed in the greater part of cytoplasm. Membrane whorl (W) is clearly recognized. Irregular dense bodies are increased and scattered throughout the cytoplasm (arrow). ×3000

Fig. 5. Enlargement of Fig. 4. A portion of cytoplasm is filled with vesicle-like sER. Mitochondria in which internal matrices lost its electron density are found. At left upper corner microvilli (mv) are seen. ×10000

Fig. 6. Higher magnification of an interstitial cell. Abundant vesicle-like sER are observed. Irregular dense bodies (arrow) contain vesicles. Two membrane whorls (W) of relatively small size are seen and one of them possess irregular dense body in its central region. ×[illegible]

Fig. 7. This structure exhibits concentric lamellae of tubular sER, however, at its central region it tends to exhibit amorphous material of low electron density. ×10000

Fig. 8. An irregular dense body is surrounded by concentric lamelle of tubular sER ×10000

Fig.4
lsc
lsc
sER
lsc

Plate IV

Fig.5

Fig.6

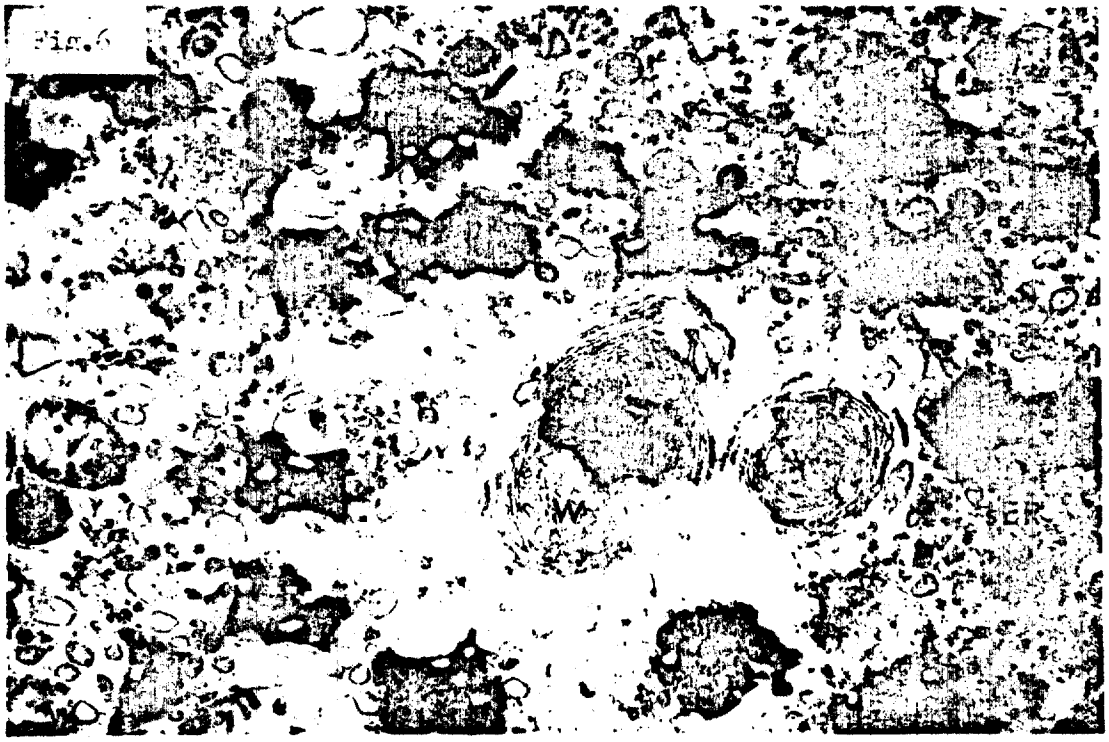

Plate V

Fig.7

Fig.6

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INTESTINAL ABSORPTION OF α-TOCOPHEROL

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(Received 28 February 1962)

DURING a study of the intestinal absorption of lipid-soluble substances an investigation was carried out into the pathway of absorption of ^{14}C-labelled α-tocopherol. Although a large volume of information appears in the literature concerning most other aspects of the tocopherols, information concerning their fate in the animal body is fragmentary. The factor of intestinal absorption has received little attention, apart from one or two indirect investigations by means of tolerance tests based on plasma tocopherol curves,[1-3] or by means of bioassays of total body tissue.[4] These methods have been subsequently criticised and shown to be unsatisfactory. Swick and Baumann[5] found that absorption of α-tocopherol is relatively fast, but no information has appeared in the literature concerning the pathway of absorption. Demonstration of the presence of α-tocopherol in lymph following oral feeding would indicate the route of absorption.

Administration of radioactive α-tocopherol and its esters in oil solution to rats has normally resulted in high faecal excretion of radioactivity.[6] A general criticism of previous work is that the so-called trace doses of 10-15 mg used in the experiments are in our opinion massive doses. These doses may have been necessary to allow subsequent analyses if low specific activity carbon-labelling or inefficient counting techniques were employed.

In the present work, ^{14}C-α-tocopherol was labelled in the methyl substituents at positions 5 and 7, by direct methylation of δ-tocopherol.* The resulting

* Adapted from a method of Dr. J. Green (Vitamins Ltd., England) kindly supplied in a private communication.

high specific activity (1 mc/mM) α-tocopherol was fed in 60 μg quantities as a single dose in 0.5 ml olive oil, to rats with cannulated intestinal lymphatic ducts. The resulting chyle was collected over a period of days or until flow ceased, using an internal collection reservoir after the method of Tasker[7]. The chyle lipids were analysed for radioactivity by liquid scintillation counting. The results (Table 1) show that incorporation of radioactivity into the chyle lipids occurs to an appreciable extent. Preliminary two-dimensional chromatography indicates that α-tocopherol passes unchanged into the lymphatic system.

TABLE 1

Animal no.	Period of chyle collection	Recovery of α-tocopherol	
	Hours	d/s	As percentage of dose fed
1	75	187	4.84
2	27	51	1.64
3	56	77.5	2.46
4	37	241	7.65
5	96	90.1	2.86

Allowing for loss of chyle due to the operative technique and overflow of the collection reservoir during overnight periods, it is evident that absorption of α-tocopherol by the lymphatic route is probably greater than indicated by the present results. A direct comparison with absorption of trace quantities of labelled palmitic acid, which is known to be well-absorbed via the lymphatic system, suggests that α-tocopherol is absorbed to the extent of 10 per cent of the administered dose.[8] Previous reports on the low incidence of tocopherol in urine were confirmed in the present work (Table 2), although the detection of some urinary radioactivity indicates that a water-soluble metabolite cannot be excluded. In view of the well-known high faecal recoveries of α-tocopherol after feeding, the results suggest that the primary absorption pathway of

α-tocopherol is via the intestinal lacteals into the chyle. Further details of the work will be published in the near future.

TABLE 2

Animal no.	Radioactivity recovered from urine after 8 days	Recovered radioactivity as percentage of dose fed
	d/s	(%)
6	11.8	0.37
7	3.0	0.09
8	10.6	0.33
9	6.7	0.20

Acknowledgements - The authors wish to acknowledge the award of a University of Birmingham Research Scholarship to one of us (P. Johnson). We also thank Miss J. Kight for valuable technical assistance and Professor A.C. Frazer for his interest.

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INVESTIGATION OF VITAMIN E IN DIFFERENT ORGANS AND TISSUE OF RABBITS FOLLOWING PER OS ADMINISTRATION WITH A TOCOPHEROL MIXTURE

Article by I. Kaludin

In order to clarify the metabolism of vitamin E in the body, different authors investigated the content in individual tocopherol fractions in different organs and tissues under special conditions of feeding and after tocopherol loading.

Bratzler & al. /1/ found only α-tocopherol in human blood. According to Harris & al. /2/, essentially only α-tocopherol can be detected in different organs of poultry. Similar results were obtained by Green & al. /3/, Bouman & Slater /4/ and Diplock & al. /5/.

Feeding experiments with swine /1, 6/ and rats /7, 8/ detected substantial storage capacities for α-tocopherol in different organs. It has been shown that the α-tocopherol content of the organs is directly dependent on nutrition. This dependence is particularly important with respect to the liver /7, 9, 10. Whiting & al. /11/ detected appreciable reserves of the vitamin in the liver of newborn sheep, goats and swing.

Following the per os administration of α-tocopherol to humans /12, 13, 14, 15/, calves /16/, cows /17, 18/ swing /19/ and hens /20/, it was determined that the concentration of α-tocopherol in blood rises to several times its initial value. After supply of the tocopherol mixture in piglets /1/ and hens /20/, strong absorption of α-tocopherol and a slighter absorption of α, β, and γ-tocopherol was observed. Absorption of α, β, γ, and δ-tocopherol was also observed in rats /21, 22, 23/ and hen turkeys /24/.

No conversion of individual tocopherols /25/ or elimination in urine /26/ has been observed under physiological conditions. H. Rosenkrantz & al. /27/ found in the digestive tract and Mervin & al. /28/ in the kidneys, tocopherol quinones in addition to the tocopherols. Similar results were obtained by Martius & Furer /29/ in experiments with tagged α-tocopherol. Earlier observations by Mackenzie & al. /30/ showed that α-tocopherol quinones and α-tocopherol hydroquinone are effective against paralysis, creatinuria and weight distrubances in deficient rabbits.

The purpose of this investigation was to obtain information, based on previous observations /31/, concerning the absorption, conversion and elimination of tocopherols, following the determination of the individual tocopherols in the blood, liver, heart, muscles, kidneys and urine of rabbits charged os with tocopherol.

Methodology:

The experiments were performed on a total of 10 rabbits with an average body weight of 3 kg. Three rabbits were used as controls, and in 3 rabbits the tocopherol content was determined in the blood and urine, following charging with a mixture of tocopherols (60 mg/animal 43% α-, 45% $\beta+\gamma$ and 12% δ-tocopherol). The animals were kept in metabolic cages and feces and urine were collected separately. Of the remaining 4 rabbits, 2 were used as controls and the other 2 treates -- as above -- per os. The livers, muscles, hearts and kidneys of these rabbits were examined. Blood samples were taken after 6 and 24 hours from the vena of the ear; urine and organs were analyzed immediately, the individual organs after homogenizing. Tocopherols were determined by paper chromatography. The preparation of the extract and the separation of individual tocopherols followed previously described methods /22/. In this method, two additional fractions, beside α-, β -, $+\gamma$-, and

δ-tocopherol, were observed. A previously described method /22/ was used in the quantitative determination of the fractions.

Results:

Results obtained with the control animals (Fig. 1 and 3) show that only α-tocopherol is present in the organs of the animals investigated. Fig. 2 and 3 indicate that 24 hours after charging with α-, β, γ- and δ-tocopherol, only α-tocopherol is detectable in the muscles, heart, kidneys and liver. Fig. 2 shows that in the 24-hour urine of rabbits charged with a mixture of tocopherols, only α-tocopherol is found. Fig. 1 and 2 further indicate that both in the control animals and in those treated with the tocopherol mixture, two additional fractions, not always clearly separated, were found (Fig. 1 - muscles and kidney).

As seen from Table 1, the blood of treated rabbits contains α-, β -, γ+ and δ- tocopherol after an absorption period of 6 hours. Twenty-four hours after treatment, β, γ - and δ-tocopherol were present in traces only. Total concentration of fractions not belonging to α-, β-, γ- and δ-tocopherol is higher in the blood of treated animals than in that of controls animals.

Two determinations in the liver yielded an average value of 2.65 mg% α-tocopherol in the controls, 6.57 mg% in rabbits treated with tocopherol.

Discussion:

As observed and described by different authors, human blood and the organs of different animals contain α-tocopherol only /1-5/. The present paper shows that these findings are valid with respect to the blood and different organs of rabbits (Fig. 1 and 3). The same results are obtained 24 hours after charging with a mixture of tocopherols (Fig. 2 and 3). It is seen from Fig. 2 and Table 1, that 6 hours after charging with a

mixture of tocopherols, β, γ and δ-tocopherol are also present in the blood. It is remarkable that β, γ and δ-tocopherol were not found in the blood of numerous rabbits after an absorption period of 24 hours. These results permit the following discussion:

1. The absorption of $\beta+\gamma$-tocopherol occurs during the first hours after charging. These fractions are not stored by the internal organs of rabbits. This is supported by the fact that in the muscles, heart, kidneys and liver (see Fig. 2 and 3) only α-tocopherol is found.
2. $\beta+\gamma$ and δ-tocopherol are rapidly converted. This assumption is supported by the fact that in the urine (Fig. 2) only α-tocopherol is eliminated.

Measured values of tocopherol in the liver are confirmed by the data for swine, sheep, goats /1, 6, 11/ and rats /7, 8, 23/, according to which liver has a special storage capability for -tocopherol. Capacity for α-tocopherol in the liver and other organs appears to be limited, because in the present work with rabbits (Fig. 2) and in previous experiments with rats deficient in vitamin E, α-tocopherols was found in the urine. Drummond & Mattill /32/ also found -tocopherol in the urine of rats after the per os administration of α-tocopherol.

The two fractions observed in the chromatograms in addition to α-, β-, γ- and δ-tocopherol (Fig. 1, 2, Table 1), appear to be intermediate products of the tocopherol metabolism. The fractions are also found in the serum and the erythrocytes of rats of various ages /32/. The fractions are still detectable in the flood of rats fasting for 72 hours, while they are no longer present in the complete blood of rats deficient in vitamin E.

Mervin & Morton /28/ found tocopherol quinones in the kidneys and explained the compounds as intermediate products of the decomposition of vitamin E. Rosenkrantx & al. /27/ found tocopherol quinones in addition

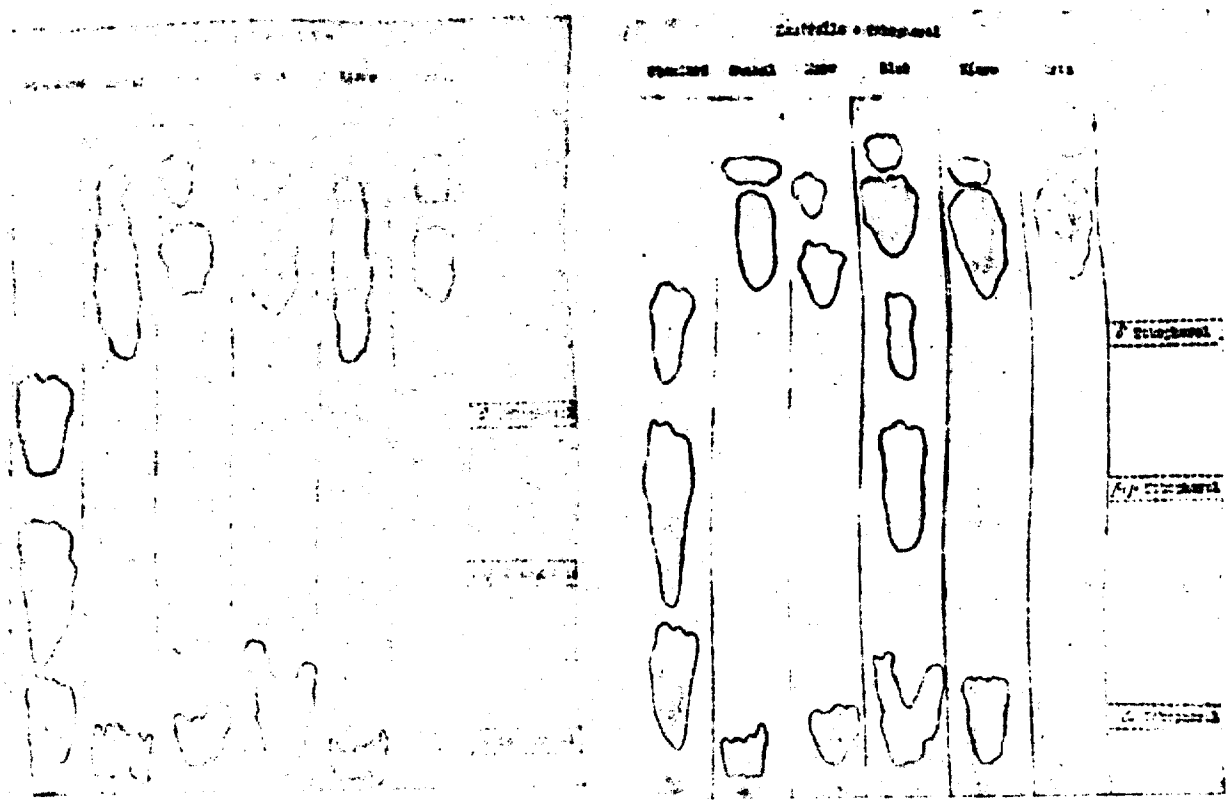
to tocopherol in the digestive tract after the per os administration of tocopherol and assumed that these represent oxidation products of tocopherol. Mackenzie & al. /30/observed in rabbits deficient in vitamin E a similar effect of α-tocopherol hydroquinone and of α-tocopherol quinone against creatinuria, paralysis and weight distrubances, as with α-tocopherol. Based on their experience with tagged tocopherol, Martius & Furer /29/ came to the conclusion that tocopherols are converted by the animal body in several stages. According to them, the conversion proceeds with preservation of the chromane ring and abbreviation of the side chain of tocopherol. Further, the chromane ring is transformed into a quinone-like compound which represents the effective factor of vitamin E. Suitable experiments are being planned in the author's institute to determine the chemical nature of the two fractions appearing in large amounts in the blood of rabbits. Results additionally support the assumption that the role of the liver with respect to α-tocopherol is mainly that of storage.

Summary:

In the present work the muscles, liver, heart, kidneys, blood and urine of normal rabbits were examined before and after the per os administration of a mixture of tocopherols (α-, β, γ-, and δ). The following was found:

1. Muscles, liver, heart and kidneys of control animals and of animals treated with tocopherol contain only α-tocopherol after an absorption period of 24 hours.
2. While the blood of control animals contains only α-tocopherol, α-, β-, γ- and δ-tocopherol may be found in the blood of rabbits treated with a mixture of tocopherols after a period of 6 hours.
3. In the 24 hour urine of charged rabbits, only α-tocopherol is eliminated.
4. In blood, urine and in the organs investigated, two fractions are found which appear in chromatograms outside of α- , β-, γ- and δ-tocopherol.

The author expresses his thanks to V. Ehrhardt, Grad. Mathematician, for the statistical evaluation.



1

2.

FIGURE 1. Tocopherol Fractions Found in Different Organs, Blood and Urine of Control Rabbits (Page 31)

FIGURE 2. Tocopherol Fractions Detectable in Different Organs, Blood and Urine of Rabbits After Per Os Treatment with -, + -, and -Tocopherol. Blood was examined after an Absorption Period of 6 Hours, Urine and the Organs After 24 Hours. (Page 33)

KEY: 1. Muscles, 2. Liver, 3. Heart, 4. Blood, 5. Kidney, 6. Urine

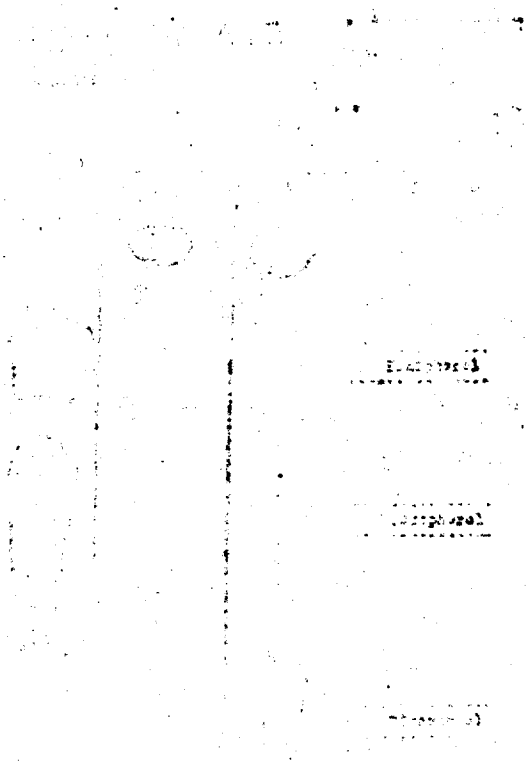


FIGURE 3. Detectable Tocopherol Fractions in the Liver of Rabbits Before and After Charging with -, + - and -Tocopherol. (Page 34)

TABLE 1. Tocopherol Content and Content in Two Fractions Found Outside of -, = - and -Tocopherol in Chromatograms of the Whole Blood of Rabbits Charged Per Os with a Tocopherol Mixture (25.8 mg -, 27.8 mg = - and 7.2 mg -Tocopherol per Animal). Values in g%. (Continued on Page 32)

Versuchs-Nr. (1)	Tocopherol: Kontrolle (3) Ges. Tocopherol (2)	davon (4) x	$\beta + \gamma$	δ	Kontrolle + Tocopherol: Ges. Tocopherol (2)	davon (4) x	$\beta + \gamma$	δ	Ges. Tocopherol: Δ gegen Kontrolle (5)	Nicht-Tocopherol (6): Kontrolle	Kontrolle + Tocopherol	Δ gegen (5) Kontrolle
1	528	528	–	–	916	644	184	88	+ 388	592	667	+ 75
2	730	730	–	–	1035	803	159	73	+ 305	493	581	+ 88
3	768	768	–	–	1189	982	168	30	+ 412	505	700	+ 195
4	597	597	–	–	886	731	119	36	+ 289	720	788	+ 68
5	536	536	–	–	945	722	123	100	+ 409	486	628	+ 142
6	550	550	–	–	870	708	87	75	+ 320	611	713	+ 102
7	631	631	–	–	927	764	105	58	+ 294	641	733	+ 92
Mittelwert (7)	629,3	629,3	–	–	965,6	744,9	135,0	65,7	345,3	578,3	687,1	+ 108,8
S	95,3	95,3	–	–	108,4	107,6	25,7	25,9	55,4	87,8	68,6	44,8
$S_{\bar{X}}$	36,1	36,1	–	–	41,1	30,8	9,7	9,8	21,0	33,2	25,2	17,0

Key 1: No. of experiment; 2. Total tocopherol; 3. Control; 4. Of this; 5. Increment with respect to control; 6. Not tocopherol; 7. Mean.

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THE INFLUENCE OF TOCOPHEROL ON THE GROWTH OF RATS

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(Received January 22, 1958)

In the previous papers, some studies on the physiological effects of tocopherol on the animals were reported, but this problem has not been fully established.

The physiological study of tocopherol was made by the authors and the influence of tocopherol on the growth of rats were presented.

EXPERIMENTAL

Test Sample and Feeds

Tocopherol used in this experiment was synthesized DL-α-tocopherol which had been recognized to be a pure material from the test on chromatography and absorption spectra.

The basal vitamin E-deficient diet is shown in Table I.

TABLE I
Vitamin E-Deficient Basal Diet

Corn starch	60
Purified lard[a]	10
Purified casein	18
Dry yeast	8
Salt mixture[b]	4
Vitamin A	50 I.U. per day per animal
Vitamin D_2 (1)	5 I.U. per day per animal

[a] Extracted from pork fat, and the tocopherol was removed by heating in the stream of air.

[b] Mixture of NaCl 1.73, $MgSO_4$·aq 5.45, NaH_2PO_4·aq 3.47, K_2HPO_4 9.54, $Ca(H_2PO_4)_2$·aq 5.40, ferric citrate 1.18 and calcium lactate 13.0

Methods

Twenty young rats (ten male and ten female rats) weighing 30--40 g were divided into five groups, and were fed on the vitamin E-deficient diet mentioned above for 40 days.

In the first group, 1 mg of DL-α-tocopherol was administered orally to each animal every day throughout the experimental period. Similarly, in the second group 5 mg, in the third group 10 mg, and in the fourth group 50 mg of the tocopherol were administered to each animal. In the fifth group which served as a control, no tocopherol was administered.

Results

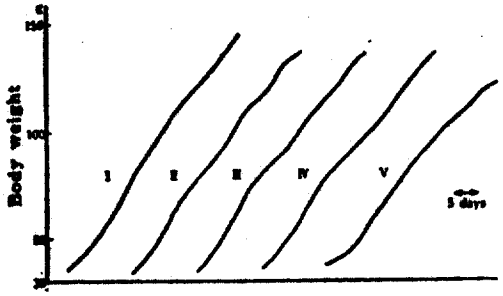

Fig. 1

In the process one animal in the first group and two in the fifth group died, but others grew well with nothing unusual in appearence.

As shown in Table II and Fig. 1, the average increase in the body weight for 40 days was 109.7 g in the first group, 101.0 g in the second, 101.3 g in the third, and 100.0 g in the fourth, but in the fifth control group, the increase was as slight as 83.5 g.

TABLE II

Average Body Weight before and after the Experiment

Group	Vitamin E administered per day per animal	Body weight (g)		
		Before	After	Increase in 40 days
	mg			
I	1	35.3	145.0	109.7
II	5	34.0	135.0	101.0
III	10	34.0	135.3	101.3
IV	50	35.3	135.3	100.0
V	0	37.5	121.0	83.5

DISCUSSION

It is definitely shown by the experiment described above that the growth of rats is promoted by the administration of DL-α-tocopherol. Regarding the amount of the tocopherol administered, a large dose is believed to exert no ill effect, but, judging from the finding that the group with 1 mg of the tocopherol showed the best growth, the daily requirement of vitamin E seems to be relatively low, about 1 mg or less.

SUMMARY

Some groups of rats weighing 30—40 g were fed on a vitamin E-deficient diet and 1—50 mg of DL-α-tocopherol per day was administered orally to each animal for 40 days. After the experimental period the growth of the groups supplemented with the vitamin was found more advanced than that of the control group, and nothing unusual in appearence was observed in the experimental animals. Therefore, vitamin E in such doses is believed to exert no ill effects on the animals.

Considering the growth response curve, the daily requirement of vitamin E for the rat seems to be 1 mg or less.[1]

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The absorption of α-tocopherol in man

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(*Received* 23 *February* 1970—*Accepted* 17 *June* 1970)

1. Physiological oral doses of DL[5-Me-^{3}H]α-tocopherol were administered to fasting subjects with various disease states, with and without steatorrhoea, and net absorption was calculated by measurement of faecal unabsorbed radioactivity. In each subject studied more than 70% of the faecal radioactivity was excreted as unchanged α-tocopherol.

2. α-Tocopherol absorption was significantly less in subjects with steatorrhoea (mean 59·4%, range 31–83%) than in those without steatorrhoea (mean 72·4%, range 51–86%). Nineteen out of twenty-four subjects with steatorrhoea absorbed less than 65% of the α-tocopherol dose, while twenty-two out of twenty-six subjects without steatorrhoea absorbed more than 65%. There was a significant correlation between the severity of steatorrhoea and α-tocopherol absorption. In a wide range of diseases steatorrhoea appeared to be the common factor associated with a decrease in α-tocopherol absorption; however, in subjects after gastric surgery α-tocopherol absorption was often low even in the absence of steatorrhoea, and this may be an important factor in explaining the high incidence of vitamin E deficiency in such patients.

3. Radioactivity in the plasma was maximal 6–12 h after dosing. Plasma radioactivity curves were significantly lower in patients with steatorrhoea than in those without steatorrhoea, but there was considerable overlap. In some patients with severe deficiency and severe steatorrhoea there was no detectable plasma response even with considerable absorption of up to 40% of the administered dose. Three patients with severe dietary deficiency of the vitamin and no steatorrhoea also showed a decreased plasma radioactivity.

4. Three subjects were studied twice, initially when deficient in α-tocopherol and a second time after repletion with α-tocopherol acetate. There was no evidence for a compensatory mechanism to increase α-tocopherol absorption in α-tocopherol deficiency.

5. A small quantity of the administered dose (almost always less than 6%) was excreted in the urine.

Deficiency of vitamin E (α-tocopherol) as judged by subnormal plasma levels is known to occur in patients with steatorrhoea (Darby, Ferguson, Furman, Lemley, Ball & Meneely, 1949; Binder, Herting, Hurst, Finch & Spiro, 1965; Losowsky & Leonard, 1967), and since vitamin E is fat-soluble it seems likely that the cause of the deficiency is diminished absorption. In most studies in man absorption has been assessed from plasma levels, which depend in part on factors other than absorption (Klatskin & Krehl, 1950; Week, Sevigne & Ellis, 1952). In the two reported studies in which measurements of unabsorbed faecal vitamin E were made (Klatskin & Molander, 1952; Rosenkrantz, Milhorat & Farber, 1953), large unphysiological doses were used and the methods of analysis were probably not specific for vitamin E. Blomstrand & Forsgren (1968*a*) have recently reported the administration of physiological amounts of ^{14}C-labelled DL-α-tocopherol acetate and ^{3}H-labelled DL-α-tocopherol in two patients and measurement of the resulting radioactivity in lymph, and suggested absorptions of from 21·3 to 28·5%.

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This paper reports the results of a study of the absorption of physiological amounts of α-tocopherol in human subjects with and without steatorrhoea by oral administration of DL[5-Me-^{3}H]α-tocopherol and measurement of resulting radioactivity in faeces, serum and urine.

MATERIALS AND METHODS

Materials. DL[5-Me-^{3}H]α-tocopherol with a specific activity of 1 mCi/mg was purchased commercially (The Radiochemical Centre, Amersham, Buckinghamshire, England). Its purity was not less than 95% on receipt, and it was never used if the purity dropped to less than 92%, as determined by thin-layer chromatography. All solvents and chemicals were Analar grade, and were used without further purification.

Plasma α-tocopherol. This was measured colorimetrically by the method of Emmerie & Engel (1939) after saponification, extraction with hexane and thin-layer chromatography as described by Bieri & Prival (1965). The lower limit of normal is taken as 500 μg/100 ml.

Faecal fat. The method was that of van de Kamer, Huinink & Weyers (1949) as modified by Frazer (1960), on 6 or 7 d balance periods, with continuous chromium marking (Rose, 1964) to correct for possible irregularity of bowel habit. The upper limit of normal is taken as 6 g/d (Dyer & Dawson, 1968).

Procedure for radioactive absorption test. A portion (15–20 μCi) of the radioactive DL-α-tocopherol, dissolved in 2 ml arachis oil containing 0·2 mg of carrier α-tocopherol, was given to fasting subjects and washed down with 100 ml milk (the α-tocopherol content of the milk was measured in our laboratory and did not exceed 20 μg/100 ml). The subjects were then fasted for a further 4 h. Faeces were collected in 3 d pools for the next 12 d. It became evident after the first twenty tests that significant activity did not appear after the first 6 d and only rarely after the first 3 d. Hence in later tests only the first two 3 d collections of faeces were analysed initially; succeeding specimens were analysed only if more than 5% of the administered dose was present in the second 3 d collection. Urine was collected in twenty-seven of the fifty subjects studied for the 6 d following administration. In twenty-nine of the fifty subjects studied, blood samples were collected into heparinized tubes at 2-hourly intervals from 4 to 8 h after the oral dose, then at 12 and 24 h and then daily to 7 d.

Faecal radioactivity. Faecal specimens were pooled as two 3 d collections and then one 6 d collection. On completion, each collection was homogenized at 13000 rev/min for 3–5 min, and 10 g portions were analysed in duplicate immediately or stored at −20° until analysed. Each portion was mixed thoroughly with anhydrous sodium sulphate powder to dry the specimen and extracted in a Soxhlet apparatus with 200 ml acetone for 2·5 h. Carrier DL-α-tocopherol (5 mg) was added to one of each pair of specimens before extraction to estimate the efficiency of recovery, and to act as an antioxidant for the small amount of radioactive material present. The lipid extract was partitioned into 100 ml light petroleum (b.p. 40–60°) from an acetone–water mixture (1:1, v/v). The light petroleum was evaporated under reduced pressure and the dry residue was taken up in 8 ml 5% pyrogallol in ethanol, placed in a boiling

water-bath under reflux and, when the solution was boiling, 2 ml 16N-KOH were added and saponification was continued for 10 min. The non-saponifiable material was extracted into 3 × 20 ml diethyl ether. The ether was evaporated and the residue was taken up in 5 ml light petroleum. Portions (0·5 ml) were measured into counting vials and evaporated to dryness. The remainder of the extract was purified on a column of Decalso F (Diplock, Edwin, Bunyan & Green, 1961) and portions of the purified eluate were measured into counting vials and evaporated. The method is basically that of the Society for Analytical Chemistry: Analytical Methods Committee (1959), with modifications as described by Edwin, Diplock, Bunyan & Green (1960). Portions of the light petroleum extract from the sample with added carrier were also chromatographed on thin-layer plates of silica gel and the distribution of radioactivity between unchanged DL-α-tocopherol and oxidation products was determined. The following solvent systems have been used for unidimensional runs: (1) ethyl acetate: hexane (15:85) (Tendille, Bats, Gervais & Gaborit, 1965); (2) diisopropyl ether: light petroleum: acetone: ethyl ether: glacial acetic acid (85:12:4:1:1) (Stowe, 1963); (3) cyclohexane: chloroform (2:1) (Skinner, Parkhurst & Alaupovic, 1964).

Plasma radioactivity. Portions (2 ml) of plasma were extracted with hexane after saponification. The hexane was transferred to counting vials and evaporated to dryness. On four occasions parallel extractions were carried out with chloroform: methanol (2:1, v/v) (Folch, Lees & Stanley, 1957) and, since this gave results identical to those with the simpler hexane extraction, the latter was used routinely. To determine the distribution of radioactivity between α-tocopherol and its metabolites in plasma, portions of hexane extracts were evaporated to dryness, taken up in successive portions (approximately 0·05 ml) of benzene and transferred quantitatively to silica gel thin-layer plates and chromatographed, using the solvents listed previously. Areas corresponding to unchanged α-tocopherol and to α-tocopherolquinone were scraped off, eluted with ethanol, transferred quantitatively to counting vials and evaporated to dryness. The remainder of the chromatogram was divided into 2 cm strips, eluted and counted similarly. Plasma counts were expressed as counts/min per ml plasma per kg body-weight per 0·15 μCi administered. These corrections are included to allow for variations in body-weight and any small differences in the total doses administered to individual patients.

Counting procedure for plasma and faecal extracts. To each dried extract were added 8 ml toluene containing 0·4% 2,5-diphenyloxazole (PPO) and 0·01% 1,4-di-[2-(5-phenyloxazolyl)]benzene (POPOP). Radioactivity was measured in a Packard Tri-Carb automatic liquid scintillation counter model 527, the minimum counts being four to five times the background of 12–14 counts/min. The efficiency was 33%. Quenching was corrected for by the external standardization channel ratio method.

Urine radioactivity. A portion (0·5 ml) of each urine specimen was diluted with 10 ml of Bray's scintillation solution (Bray, 1960) and counted as above.

Calculation of net absorption. It was assumed that any radioactive α-tocopherol not excreted in the faeces had been absorbed. Net absorption was calculated by

subtracting the faecal radioactivity from the total radioactivity administered and expressing this difference as a percentage of the total dose administered.

Statistical methods. Means of groups were compared by Student's *t* test and correlation coefficients calculated with the Bravais–Pearson coefficient of linear correlation.

Table 1. *Subjects studied*

Diagnosis	No. of patients	No. with steatorrhoea
Cirrhosis	16	7
Primary biliary cirrhosis	2	2
Post-gastrectomy	8	4
Gluten-sensitive enteropathy	5	3
Crohn's disease	4	4
Chronic pancreatic dysfunction	3	2
Small-bowel resection	1	1
Jejunal diverticula	1	1
Mild iron or folic acid deficiency	3	0
Obesity	1	0
Thyrotoxicosis	1	0
Hyperparathyroidism	1	0
Diabetes mellitus	1	0
Paget's disease of bone	1	0
Dietary osteomalacia	1	0
Duodenal ulcer	1	0
Total	50	24

Subjects studied. Fifty subjects were studied, all of whom were undergoing investigation for malabsorption in a metabolic unit. The diagnoses are shown in Table 1. The patients with cirrhosis all had the diagnosis proved by liver biopsy. Of the five patients with gluten-sensitive enteropathy, two had previously been shown to have steatorrhoea and had been successfully treated with a gluten-free diet for 2 and 3 years and did not have steatorrhoea when studied. The other three patients with steatorrhoea had a subsequent satisfactory response to a gluten-free diet. The three patients with chronic pancreatic disease had the diagnosis proved at autopsy or at laparotomy. The diagnosis for the four patients with Crohn's disease was proved by histological examination. The subject with osteomalacia had been taking a very poor diet; no disease was discovered despite extensive investigation. The remaining patients with diagnoses other than those given above, and who were all shown not to have steatorrhoea, have been grouped together as control patients. Three of these subjects, who were vitamin E-deficient when first studied, were repleted with 200 mg α-tocopherol acetate orally per day for 3 months and then had the absorption test repeated. The oral supplements were omitted on the day the absorption test was performed.

RESULTS

Plasma α-tocopherol levels

Plasma α-tocopherol levels were significantly lower ($P < 0.025$) in the patients with steatorrhoea, mean 477 μg/100 ml, than in those without steatorrhoea, mean 650 μg/100 ml (Fig. 1).

Net absorption of α-tocopherol

The net absorption of α-tocopherol in patients with and without steatorrhoea is shown in Fig. 2. The mean absorption for those with steatorrhoea was 59·4%, range 31–83%, and for those without steatorrhoea 72·4%, range 51–86%. While a considerable overlap existed, the means for the two groups were significantly different ($P < 0.001$).

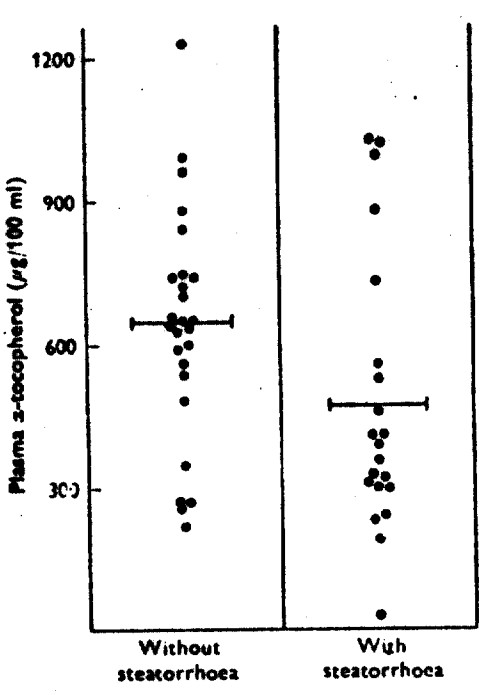

Fig. 1

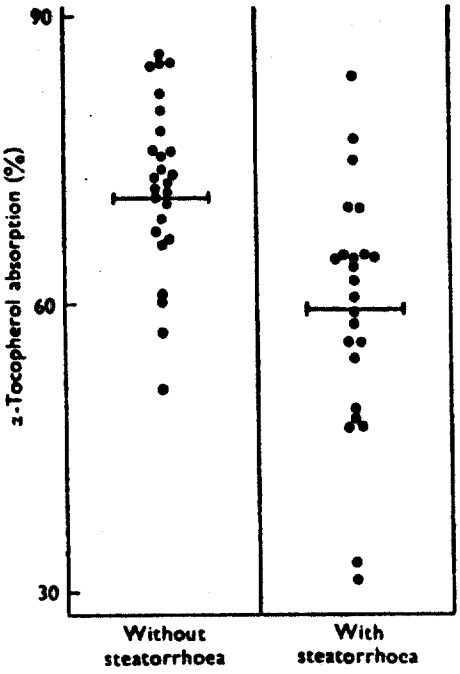

Fig. 2

Fig. 1. Plasma α-tocopherol levels in human subjects with and without steatorrhoea. The values for three subjects who had been given supplements of α-tocopherol were excluded.

Fig. 2. α-Tocopherol absorption in human subjects with and without steatorrhoea.

The net absorption of α-tocopherol in the various disease states studied is shown in Fig. 3. There was no significant difference in the absorption between the control group and the patients with cirrhosis of the liver without steatorrhoea ($P < 0.1$), but in patients with cirrhosis (excluding primary biliary cirrhosis) and steatorrhoea the absorption was significantly less than in either of the above two groups ($P < 0.001$ and $P < 0.005$ respectively). The two patients with primary biliary cirrhosis had steatorrhoea (12·9 and 7·1 g faecal fat/d) and α-tocopherol absorptions of 49 and 64% respectively. The two patients with treated coeliac disease had absorptions well up in the non-steatorrhoea range, and the values for the three untreated patients, all of whom had steatorrhoea, were much lower. Two of the three patients with chronic pancreatic disease had severe steatorrhoea (37 and 38 g faecal fat/d) and both had low

α-tocopherol absorptions (47 and 56%). The third patient, although without steatorrhoea, had an absorption that was nevertheless lower than any of those in the control group. The four patients with Crohn's disease all had steatorrhoea, and in three the absorptions were much less than the lowest value in the group without steatorrhoea. The fourth patient had a faecal fat excretion of 11·9 g/d but absorbed over 80% of the vitamin. The absorption of α-tocopherol in patients after gastrectomy tended to be lower than in the other groups without steatorrhoea. No patient after gastrectomy had

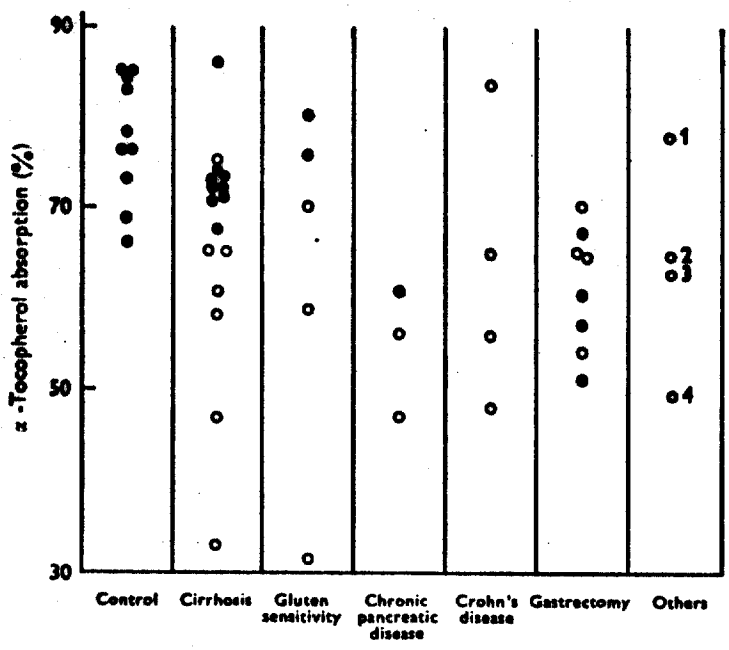

Fig. 3. α-Tocopherol absorption in human subjects with (○) and without (●) steatorrhoea separated according to diagnosis. The diagnoses in the last column were small-bowel resection (1), primary biliary cirrhosis (2, 4), and jejunal diverticula (3).

severe steatorrhoea, the highest faecal fat levels being 6·1, 7·3, 7·9 and 10·0 g/d and, although the α-tocopherol absorption was less than 70% in all of these, the other four post-gastrectomy patients without steatorrhoea had α-tocopherol absorptions just as low. In one patient with small-bowel resection and a faecal fat excretion of 10·8 g/d, the α-tocopherol absorption was 77%, while in the patient with multiple jejunal diverticula, who had mild steatorrhoea (7·0 g faecal fat/d), 62·4% of the vitamin was absorbed.

The results for three patients in whom the absorption test was repeated after repletion with α-tocopherol acetate are shown in Table 2. It is seen that in all three patients the absorption was similar in the deficient and repleted states.

The relation between the percentage absorption of α-tocopherol and the daily faecal fat output in all patients studied is plotted in Fig. 4 and, although the scatter was wide, there was a significant correlation ($r = 0{\cdot}527$, $P < 0{\cdot}001$). Only four of

twenty-six subjects with a faecal fat excretion of less than 6 g/d had an α-tocopherol absorption of less than 65%, while nineteen of twenty-four subjects whose faecal fat excretion was greater than 6 g/d absorbed less than 65%.

Table 2. *Absorption of α-tocopherol by human subjects before and after repletion with α-tocopherol*

	Deficient subjects		Repleted subjects	
Condition of subject	Serum α-tocopherol (μg/100 ml)	α-Tocopherol absorption (%)	Serum α-tocopherol (μg/100 ml)	α-Tocopherol absorption (%)
Cirrhosis (with steatorrhoea)	330	61·0	1340	58·0
Gluten-sensitive steatorrhoea	234	64·4	1450	59·2
Mild folic acid deficiency (no steatorrhoea)	356	79·8	1480	78·0

Faecal radioactivity

In thirty-five of the fifty subjects, less than 6%, and in forty of the fifty subjects less than 10%, of the administered dose appeared after the 3rd day. The remaining ten subjects included six with steatorrhoea (one with cirrhosis, two Crohn's disease, one gluten sensitivity, one chronic pancreatic disease and one post-gastrectomy), and four without steatorrhoea (one with thyrotoxicosis, one diabetes, one chronic pancreatic disease and one cirrhosis). The late excretion of administered radioactivity in these subjects was as high as 30% of the dose in four of them, 21% in one, and in the other five between 10 and 20%. In only two subjects did more than 2% of the administered radioactivity appear later than the 6th day; one had chronic pancreatic disease without steatorrhoea and excreted 6% after the 6th day and the other had gluten-sensitive enteropathy with steatorrhoea and excreted 10% after the 6th day.

Thin-layer chromatography of the faecal extracts showed that not less than 70% of the unabsorbed radioactivity was unchanged α-tocopherol in any patient. About 10–15% of the excreted radioactivity was in a single distinct spot with a R_F value in several solvent systems (Tendille *et al.* 1965; Stowe, 1963; Skinner *et al.* 1964) similar to α-tocopherolquinone, with which it was identified. The remainder was spread evenly along the rest of the thin-layer plate.

Plasma radioactivity

The peak of plasma radioactivity occurred between 6 and 12 h after the oral dose of labelled α-tocopherol in every subject in whom plasma curves were examined. A second peak was never found and the shapes of all the plasma curves were similar.

Thin-layer chromatography of plasma extracts from twenty-nine subjects showed that more than 80% (range 80–96) of the radioactivity in plasma was present as unchanged α-tocopherol. The remaining radioactivity was mainly α-tocopherolquinone and a smaller (up to 10%) unidentified peak of radioactivity was present at the solvent front.

The mean plasma curves from sixteen subjects without steatorrhoea and thirteen subjects with steatorrhoea are shown in Fig. 5. While there was a considerable overlap,

there was a significant difference between the two groups at each point on the curve except at 4 h.

The relation between the plasma radioactivity at 8 h and the absorption of the vitamin

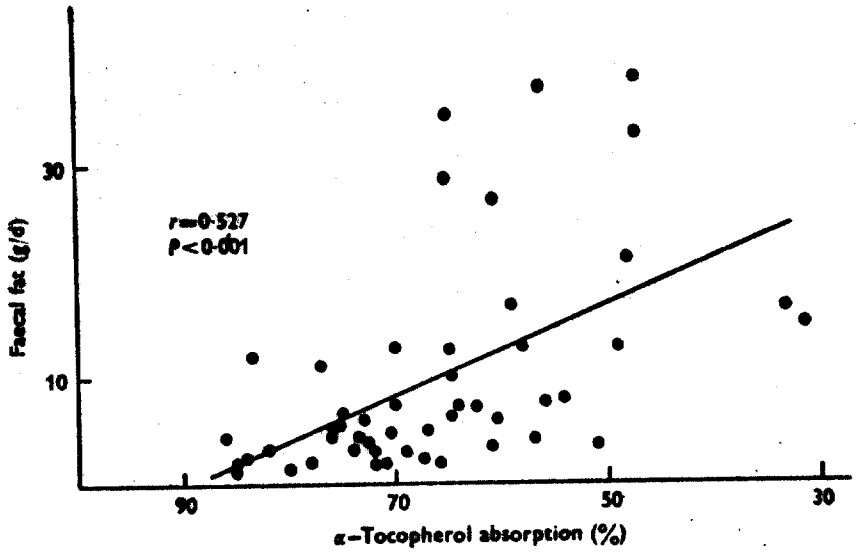

Fig. 4. Relationship between faecal fat excretion and α-tocopherol absorption in human subjects with and without steatorrhoea. The diagonal line is the calculated regression line ($y = 37{\cdot}3 - 0{\cdot}42x$).

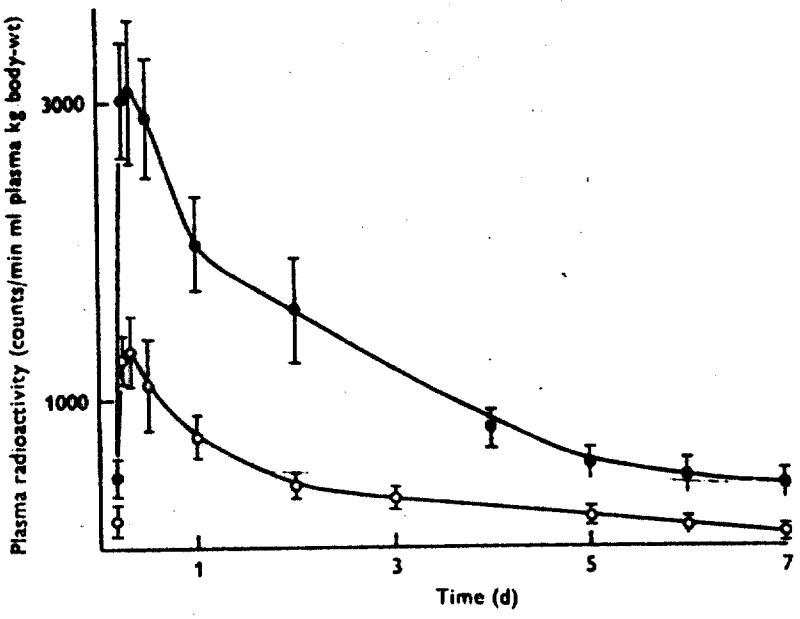

Fig. 5. Mean plasma radioactivity curves for sixteen patients without steatorrhoea (●—●) and thirteen patients with steatorrhoea (○—○). The vertical bars are the standard errors of the means. The difference was significant at all times ($P < 0{\cdot}05$) except at 4 h ($P < 0{\cdot}1$). Plasma radioactivity values are corrected for total dose administered.

is shown in Fig. 6 ($r = 0{\cdot}627$, $P < 0{\cdot}001$). Three patients with low absorptions, between 31 and 48%, failed to show any plasma radioactivity, and all three were extremely severely depleted of the vitamin as judged by plasma levels, and all had gross steatorrhoea. Similarly, three patients with severe dietary α-tocopherol deficiency but without steatorrhoea seemed to form a distinct group lying above the others on the graph, the plasma peak never reaching expected levels.

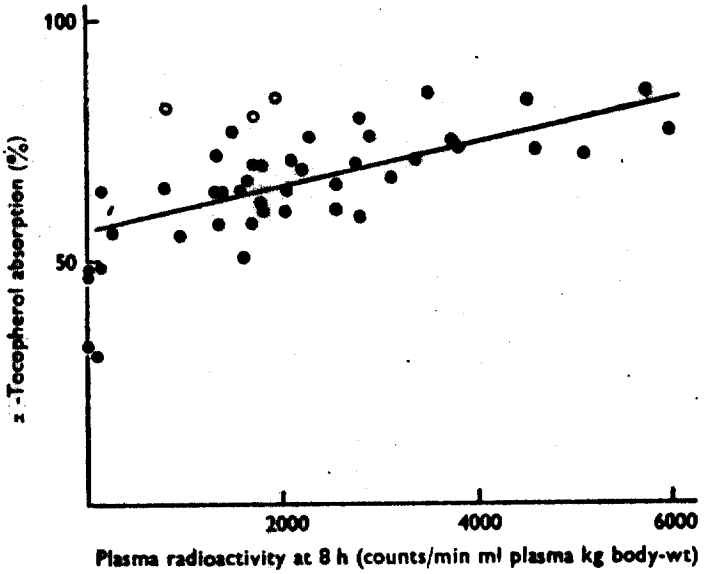

Fig. 6. Relationship in human subjects between plasma radioactivity at 8 h after dosing with α-tocopherol and α-tocopherol absorption. ○, patients with dietary deficiency of α-tocopherol; ●, patients with an adequate dietary intake of α-tocopherol. Plasma radioactivity values are corrected for total dose administered. The diagonal line is the calculated regression line ($y = 57{\cdot}2 - 0{\cdot}0045x$); $r = 0{\cdot}627$; $P < 0{\cdot}001$.

Urine activity

In twenty-five out of twenty-seven subjects the total urine excretion did not exceed 6% of the administered dose and, in seventeen out of twenty-seven, it was less than 4%. Two subjects, one with Crohn's disease and steatorrhoea and the other a gastrectomy patient without steatorrhoea, excreted 7·2 and 7·5% of the oral dose respectively. Of the excreted radioactivity, more than 75% was in the urine excreted during the 48 h following the oral dose and, by the 6th day, less than 0·1%/d was being excreted. The nature of this urine radioactivity was not studied further.

DISCUSSION

Completeness of absorption

That the absorption of α-tocopherol is incomplete is suggested by many studies in animals (Krishnamurthy & Bieri, 1963; Simon, Gross & Milhorat, 1956) and a small number of studies in man (Klatskin & Molander, 1952; Rosenkrantz *et al.* 1953).

This appears to be true for all the fat-soluble vitamins. Thompson, Lewis & Booth (1966) found 78% absorption of cholecalciferol, Blomstrand & Werner (1967) 6–60% absorption of retinol, and Blomstrand & Forsgren (1968*b*) 60% of phylloquinone. Blomstrand & Forsgren (1968*a*) studied the absorption of vitamin E in two patients with cannulated thoracic ducts. In one subject with a gastric carcinoma absorption of DL-α-tocopherol acetate was 21·3% and in the other patient with chronic lymphatic leukaemia the absorption of DL-α-tocopherol acetate was 25% and the absorption of DL-α-tocopherol was 28·5%. There are several possible reasons for these absorption results being lower than the values reported in this study. It has not been established that α-tocopherol absorption is exclusively by the lymphatic route; lymph collection via the thoracic duct in man may not be complete owing to anatomic variations (Kayden, Senior & Mattson, 1967), and the same authors stated that recoveries of labelled fatty acids in lymph triglycerides in man are low, ranging from only 25–60%, and malabsorption does not appear to have been excluded in these ill patients. Furthermore, the present study has estimated net absorption from all levels of the gastrointestinal tract, and it seems possible that absorption of the vitamin could continue for longer than the 24 h studied by Blomstrand & Forsgren (1968*a*).

There is evidence that α-tocopherol is excreted in the bile (Klatskin & Molander, 1952; Schmandke & Proll, 1964) and this could be re-absorbed and constitute an entero-hepatic circulation. However, Mellors & Barnes (1966) failed to detect radioactivity in bile from rats given oral doses of ^{14}C-labelled α-tocopherol, Schmandke & Proll (1964) found only 0·8% of an intravenously administered dose appearing in the bile and Klatskin & Molander (1952) found no increase in bile levels following oral administration of α-tocopherol in man. Thus, the biliary excretion and entero-hepatic circulation of newly absorbed α-tocopherol seem unlikely to have a significant effect on the net absorption as we have determined it.

Mechanism of absorption

Little is known about the mechanism of absorption of α-tocopherol. However, it does appear that the free alcohol is absorbed as such, while the ester is hydrolysed during or soon after absorption, for several studies have shown that increases in plasma levels obtained after oral doses of the acetate ester are due to the presence of the free alcohol (Filer, Wright, Manning & Mason, 1951; Pelkonen, 1963). Furthermore, Blomstrand & Forsgren (1968*a*) showed in a patient who received an oral dose of ^{14}C-labelled DL-α-tocopherol acetate that over 90% of the radioactivity in the thoracic duct lymph was present as the free alcohol. Wiss, Bunnell & Gloor (1964), however, suggested that α-tocopherol acetate might be partly absorbed as the unchanged ester and partly as the free alcohol resulting from hydrolysis of the ester in the intestine, although, in contrast, orally administered vitamin A, whether free or esterified, appears in the intestinal mucosal cells (Ganguly, Krishnamurthy & Mahadevan, 1959) and thoracic duct lymph (Goodman, Blomstrand, Werner, Huang & Shiratori, 1966; Blomstrand & Werner, 1967) almost entirely as the palmitate.

That α-tocopherol absorption is linked with fat absorption is clear from various reports. Darby *et al.* (1949) and Losowsky & Leonard (1967) found low plasma levels

of the vitamin in patients with steatorrhoea, and Hashim & Schuttringer (1966) and Filer *et al.* (1951) found no increase in plasma levels after oral administration of the vitamin to subjects with severe steatorrhoea. These results, taken with the findings now reported, support the view that α-tocopherol absorption is reduced in patients with steatorrhoea. It has been suggested (Pomeranze & Lucarello, 1953) that α-tocopherol absorption follows fat absorption closely, that the route of absorption is probably lymphatic (Blomstrand & Forsgren, 1968*a*; Johnson & Pover, 1962), and that most of the absorbed vitamin is located in the chylomicrons. McCormick, Cornwell & Brown (1960) showed that the increment in serum tocopherol from newly absorbed α-tocopherol is found first in the chylomicrons and Sf 10–400 lipoproteins. The transport of newly absorbed α-tocopherol in chylomicrons in the lymphatics would be similar to that for vitamin A (Goodman *et al.* 1966; Blomstrand & Werner, 1967) and cholecalciferol (Avioli, 1969) and also for the absorption of dietary triglycerides (Senior, 1964; Kayden *et al.* 1967). In the rare syndrome of abetalipoproteinaemia, in which there is a defect of chylomicron formation and an absence of fat absorption into lymphatics (Ways, Parmentier, Kayden, Jones, Saunders & Rubin, 1967), there also appears to be an absence of α-tocopherol absorption, as judged by the response of plasma α-tocopherol levels to prolonged oral administration of α-tocopherol acetate (Kayden & Silber, 1965). The fact, however, that some fat absorption does occur in these patients, possibly via the portal vein (Ways *et al.* 1967), might suggest that unless it is entirely taken up by the liver in the first circulation α-tocopherol cannot be absorbed into the portal vein. However, Dodge, Cohen, Kayden & Phillips (1967) and Muller & Harries (1969), using large doses of water-miscible α-tocopherol in patients with abetalipoproteinaemia, did produce some response in plasma levels. Perhaps, therefore, if lymphatic absorption is blocked, absorption of water-miscible preparations by the portal route assumes importance. Furthermore, Schmandke (1967) demonstrated higher post-absorptive α-tocopherol levels in portal as compared with peripheral blood in rats, suggesting some degree of absorption via the portal vein.

The lack of a closer correlation between α-tocopherol absorption and faecal fat in our patients may have been caused in part by the diversity of their disease states, which may have had different effects on α-tocopherol absorption and faecal fat. Furthermore, the method we have used for performing the absorption test may explain the overlap between patients with and without steatorrhoea in that we have used tracer doses of the vitamin, which may not require maximal absorptive activity. The presence of bile is thought to be necessary for normal absorption of vitamin E (Klatskin & Tisdale, 1957; Greaves & Schmidt, 1937; Brinkhous & Warner, 1941) and this may be related to the role of bile salts in the formation of lipid micelles for fat absorption (Hofmann & Borgström, 1962, 1964). Studies in animals suggest that no one part of the gastro-intestinal tract is responsible for α-tocopherol absorption (Sternberg & Pascoe-Dawson, 1959; Desai, Parekh & Scott, 1965) and sufficient information is not yet available for human subjects to decide if any specific site exists.

Plasma curves

The plasma content of newly absorbed vitamin E at any time will depend on several factors, the most important of which are probably the rate of absorption, the rate of transfer to the tissues and the quantity of transporting lipoprotein present in the plasma (Davies, Kelleher & Losowsky, 1969).

The time of peak plasma activity, 6–12 h after administration, found in our studies is very similar to that found by other authors, who used large doses of unlabelled vitamin E (Filer *et al.* 1951; Week *et al.* 1952), labelled cholecalciferol (Thompson *et al.* 1966) and unlabelled vitamin A (Harris, 1960). Since the height of the plasma peak depends on other factors as well as absorption, it is perhaps to be expected that there would not be a close correlation between total absorption and the height of the plasma peak, and a similar lack of close correlation has been observed after oral doses of physiological amounts of cholecalciferol (Thompson *et al.* 1966). This is illustrated by the three of our patients without steatorrhoea, who had a vitamin E deficiency of dietary origin and whose plasma peaks were lower than in other patients with similar net absorptions. It seems likely that in these three patients there was a rapid transfer of newly absorbed vitamin to the tissues, reducing the accumulation in the plasma; also in animal experiments (Kelleher, Davies & Losowsky, 1969) the uptake of α-tocopherol by the tissues has been shown to be very much greater in deficient than in supplemented or normal animals. Similarly, in some of the subjects with severe steatorrhoea and severe deficiency, as shown by very low plasma α-tocopherol levels, there was no detectable plasma radioactivity even though as much as 31–47% of the dose was absorbed. The absence of an increase in plasma α-tocopherol concentration in patients with severe steatorrhoea has also been shown by other workers using large oral doses of the vitamin (Filer *et al.* 1951; Hashim & Schuttringer, 1966). While absorption is diminished in such patients, it is still considerable, and the rapid uptake of the absorbed vitamin by severely depleted tissues may be even more important.

In patients with steatorrhoea the concentration of low-density lipoproteins is often reduced (Lloyd, 1969) and, as these are the major transporting proteins for α-tocopherol (McCormick *et al.* 1960; Davies *et al.* 1969), it may well be that this reduction would contribute to lowered plasma levels.

Absorption in disease states

Based on their measurements of faecal excretion, Klatskin & Molander (1952) made the suggestion that the absorption of α-tocopherol is greater in patients with cirrhosis than in control convalescent subjects, but in a later paper (Klatskin, 1954) one of the same authors suggests that the relatively low faecal excretion may be due to increased intestinal destruction of α-tocopherol in patients with cirrhosis. Increased absorption in such patients was not found in our study and, in the absence of steatorrhoea, there was no significant difference between the absorption of α-tocopherol by subjects with cirrhosis and by the control group of subjects. In patients with cirrhosis and steatorrhoea, the absorption of α-tocopherol was diminished, as it was in those with steatorrhoea accompanying other diseases.

In patients after gastrectomy, steatorrhoea was never severe and was present in only four of the eight subjects, yet only two of the eight subjects had vitamin E absorptions within the control range. This suggests that impaired absorption of α-tocopherol may be common in such patients, even in the absence of steatorrhoea, which is also suggested by the findings of Gounelle, Marnay & Blondin (1953), who showed that the plasma α-tocopherol tolerance curve is significantly lower after gastrectomy than in normal subjects. The poor absorption could be partly due to an altered dietary habit, since Pomeranze & Lucarello (1953) produced evidence that α-tocopherol absorption is diminished in patients on a low fat intake, but there are, of course, other disturbances of gastro-intestinal physiology after gastrectomy (Lundh, 1962), that may well affect the absorption of vitamin E. Losowsky, Leonard, Kelleher & Pulvertaft (1967), in a series of over 1000 patients after gastric surgery, showed that 26% had evidence of deficiency of vitamin E. While diminished absorption may be a factor in causing deficiency, dietary intake may also be low in these subjects (Barker, Malm & Roemtsma, 1960).

Absorption in depleted subjects

In the three patients whom we have studied both in a depleted state and then 2–3 months later after repletion with α-tocopherol, the absorption was similar on both occasions. This seems to apply whether or not there is steatorrhoea, since two of the subjects had steatorrhoea (which did not alter during the period) whereas the third did not have steatorrhoea. The results at first sight appear at variance with those of Schmandke (1967), who has suggested that absorption of vitamin E is less efficient in deficient than in normal rats, and of Desai *et al.* (1965) who found similar results in chicks. However, Schmandke used the liver level of radioactivity to assess absorption, and this method, in our experience (Kelleher *et al.* 1969), may not be valid in deficient animals. Desai *et al.* (1965) measured the uptake of radioactivity in ligated intestinal loops over short time intervals (5 min), and it is unlikely that such studies would indicate the net absorption. It appears, however, from our results that in man when there is a deficiency of α-tocopherol there is no compensatory mechanism to increase the efficiency of its absorption, as there is for iron absorption in iron deficiency (Bothwell, 1968).

Excretion in the urine

The small percentage of the oral dose excreted in the urine by the subjects in this study, 67% of whom excreted less than 4% of the administered dose in the urine, is in agreement with the results of other workers with animals (Simon *et al.* 1956; Johnson & Pover, 1962). This suggests that urine is not an important excretory pathway for absorbed α-tocopherol. These results contrast with those of Sternberg & Pascoe-Dawson (1959), who found a cumulative urine excretion of 37% in rats given an oral tracer dose of α-tocopherol.

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Effect of alpha-tocopherol administration on the growth of transplanted tumors in rats

by N. A. Kochur, N. I. Goldshteyn, I. I. Ivanov, S. K. Buzas, and B. N. Tarusov

Much work has been dedicated to the problem of the role of inhibitors of free-radical reactions in the development of malignant neoplasm; however, there does not yet exist a complete clarification of this problem (Burlakova, 1968). Interesting are studies in which on the basis of a presentation about the free-radical mechanism of carcinogenesis undertaking attempts at inhibition of growth of tumors by various synthetic antioxidants and showing a greater inhibiting activity than phenol and its products in the development of leukemia, leukosis, and ascitic carcinoma of Erlikh (Emanuel', Lipchina, 1958; Frankfurt and others, 1963).

From matter possessing antioxidant properties, in the organism of animals first let us turn our attention to tocopherol. Although its biological role is far from being completely known, it is possible now already to talk about two possible functions of the tocopherol in the cell. So all of the large amount of information about the role of tocopherol in the processes of electron transfer and phosphorylation passing through the mitochondria are accumulated. In 1934 was discovered for the first time the increase in consumption of oxygen by the tissue specimens from muscles of rabbits with vitamin E deficiencies. In the future the place of alpha-tocopherol in the processes of cellular respiration will be documented by experiments in vitro with purified enzyme systems (Gorkin, 1959).

It has been known for a long time that in cancer cells ATP synthesizes fundamentally as a result of glycolysis. For some time past there have appeared many indications that in the process of malignization the activity of reduction-oxidation enzymes of the respiratory chain (cytochrome-oxidase, succinate dehydrogenase, NADF-diaphorase, and others) changes, which at the same early stage of cancerogenesis intensly expresses the decrease of their activity (Raykhlin, 1967). It is possible that it involves damage to the mitochondrial membrane and changes in its permeability that tend away from the normal dispersion cofactors, ATP, phosphor, toward a change in permeation of the substrates, etc.

This is not to exclude the possibility that tocopherol is one of the cofactors, which undergoes the changes in the tumorous cells. A study of tocopherol is especially interesting in its bonds with the developed concept of its participation in regulating the level of free-radical reactions, taking place in the cells and intensifying or inhibiting them durign diverse pathology. But immediate investigations of the effect of tocopherol of the processes taking place in the are complicated because not one of the applicable methods of injection could guarantee its complete assimilation. Therefore, the method of injection of large doses of tocopherol for the creation of a reserve of it within the organism is used (Drokova, 1958).

We studied the effect of alpha-tocopherol and at the same time its acetate, not possessing the anti-oxidizing qualities, in growth rate of some implanted tumors. In order to ascertain whether or not assimilation of the injected tocopherol in the organism takes place,

after the injection we controlled the level of inhibitors and lipids extracted from the livers of the test animals. White mongrel male rats were used in all of the experiments, weighing from 80 to 120 g. Lipids were extracted by the Folch method. Registering of chemiluminescence was carried out by the photomultiplier FEU-42, working in the quantometric regime. The temperature in the cuvette with lipids was maintained at a constant 85°. The preparation of alpha-tocopherol from the firm "Serva" was injected in 300 mg/kg intraperitoneally on the 2nd, 5th, 10th, 13th, and 15th days after implantation of the tumors.

After the injection of alpha-tocopherol into the tumorous animals the period of induction in the kinetic curve of chemiluminescence of lipids of the liver was increased, as they say of the increase in its inhibitors (Fig. 1). Particularly interesting is the fact that the injection in the very same manner of alpha-tocopheryl acetate does not show itself in experiments on fatty acids with antioxidant properties, likewise causing an increase of the induction period during the oxidation of lipids of the liver which, however, is expressed most weakly when connected with the injection of alpha-tocopherol (Fig. 1).

In these preliminary experiments, on animals with implanted sarcoma 45, therapeutic effects were recorded- inhibition rate of growth of the tumors and increase in length of life of the animals. Subsequent experiments used the implanted strains of carcinosarcoma Walker, sarcoma M-1, sarcoma 45, and ascitic OYa, transplanting every tumor of 25-30 animals. Alpha-tocopherol and alpha-tocopheryl acetate from the Moscow Experimental Vitamin Factory were used. The dosage of the injected preparation was increased to 3000 mg/kg in one injection, as the alpha-tocopheryl acetate in smaller levels developed antioxidant properties in the organism, than the alpha-tocopherol. Besides this, the alpha-tocopherol of the Moscow factory is more weakly antioxidant than the preparation of the firm "Serva". The toxic effect of the injection of larger dosages of tocopherol are not shown (Drokova, 1958).

Seven-fold (with intervals in one day) intraperitoneal injections of an oily preparation of alpha-tocopheryl acetate brought about the inhibition of the growth of sarcoma 45 and Walker's carcinosarcoma (Fig. 2); during this period, the experimental animals' lives were extended. Analogous results were obtained for animals with E-hypervitaminosis. Hypervitaminosis was created by injecting an oily preparation of alpha-tocopheryl acetate intramuscularly in the course of the two weeks before the implantation of the tumors. Until the implantation each animal was injected with nearly 1000 mg of the preparation. After the implantation the injections continued intraperitoneally.

Subsequently, in order that the injected preparation be more uniformly absorbed, it was emulsified, by mixing alpha-tocopherol (or alpha-tocopheryl acetate) with olive oil in a proportion of 3:2 (by weight), adding like amounts of an isotonic solution of NaCl, consisting of 0.1% emulsifier (gelatine), and agitation the mixture for five minutes in a vibratory mill. The control was olive oil, treated in the same manner.

The inhibiting effect on the growth of all the studied strains of tumors increased, although the dosages used were smaller, up to 400 mg/kg in one injection (Fig. 2a, curve 2).

Six animals (22%) completely recovered from 27 tumors (sarcoma 45); after three months these animals were killed and metastasis was not discovered. The control injection of emulsified olive oil

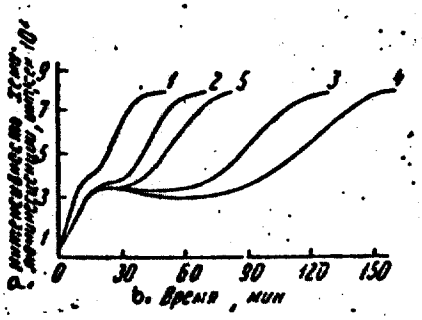

Figure 1.

Kinetics of chemiluminesce of lipids of the livers of rats after introduction of alpha-tocopherol and alpha-tocopheryl acetate (t= 85° C):
1- control; 2,3,4- respectively, on the 2nd, 10th and 13th day after beginning of injection of alpha-tocopherol; 5- on the 13th day after the beginning of alpha-tocopheryl acetate.
a- intensiveness of the chemiluminescence, impluses/sec·10^4.
b- time, minutes

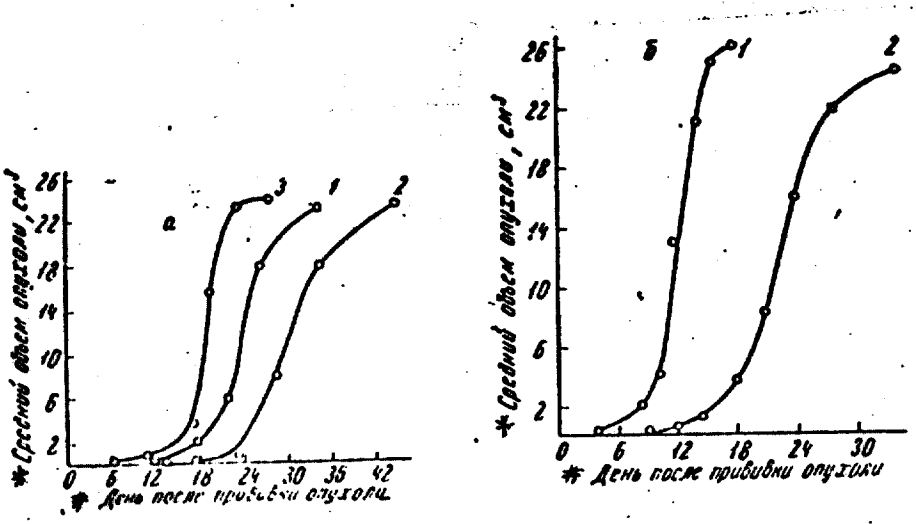

Figure 2.

Growth of tumors in rats with implanted sarcoma 45: a-after injection of the butyrous preparation of alpha-tocopherol (alpha-tocopheryl acetate):
1- without preliminary emulsification (3000 mg/kg in one injection);
2- emulsified 400 mg/kg in one injection; 3- control;
b- without the injection of tocopherol:
1- rats with vitamin E deficiency; 2- rats supported by the usual ration.
*- average size of tumor, cm^3
#- days after implantation of tumor

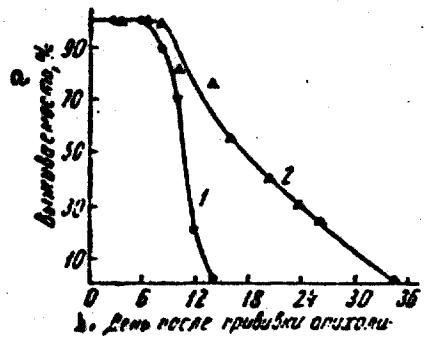

Figure 3.

Survival rate of rats with implanted ascitic OYa:
1- control; 2- after injection of emulsified alpha-tocopherol (400 mg/kg in one injection).
a- survival rate, %.
b- days after implantation of tumor.

somewhat stimulated growth of the tumors; however, these data are questionable. The fact of the difference between the experimental and control (without reactions) groups of animals was calculated on the 20th day of the experiment (sarcomas 45 and M-1) and on the 16th day (Walker's carcinosarcoma). In all the cases, the difference was authenticated ($P < 0.05$). The difference between the effects of the emulsified tocopherol and tocopheryl acetate was not discovered.

The interest in studying the speed of growth of tumors of animals with E-hypovitaminosis was described. For the creation of avitaminosis E in rats they were kept on a special diet for 150 days (Kudryashov, 1940). Since from the growth of the animals we studied the growth characteristics of the implanted sarcoma 45 (Konoplev, 1960), in the control group animals of the same age but subsisting on the usual ration were used. Although there was a slower growth of sarcoma 45 in the adult rats, in the group of animals with E-vitaminosis the tumors developed much earlier than in the young rats in the preceding experiments (Fig. 2b).

In this manner, injections of alpha-tocopherol and alpha-tocopheryl acetate caused inhibition of the development in all strains of experimental tumors used by us (sarcomas 45 and M-1, Walker's carcinosarcoma, and ascitic OYa) (Fig. 3). This therapeutic effect is not connected with the toxicity of the injected preparation, as even prolonged injection of large doses of tocopherol into rats with natural E-hypervitaminosis did not cause symptoms of depression or death in the animals.

The results we obtained in certain levels correlates well with the results of studies indicating that simultaneous injection of tocopherol with carcinogens leads to recovery from almost all tumors, developing from the effect of a carcinogen (Bonmasser et al, 1968). The injection of synthetic antioxidants - propyl gallate- does not cause such an effect, although during this there is an increase in the number of animals living until the 28th week. This is also affirmation that matter possessing antioxidant properties shows a therapeutic effect on the tumorous animals. Apparently, many of the antioxidants as biologically active substances are capable of normalizing the reduction-oxidation processes taking place in the organism. The effect of tocopherol on the growth of tumors is connected possibly also with its participation in the processes of tissue respiration and oxidative phosphorylation.

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The absorption, storage, and metabolism of α-tocopherol-C^{14} in the rat and chicken

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SUMMARY

α-Tocopherol-C^{14} was administered orally to rats and chicks, and its distribution in the body and rate of excretion were determined at short intervals up to 24 hr and at longer intervals to 21 days. The radioactivity in all tissues was identified almost exclusively as unchanged α-tocopherol except after 21 days, when trace amounts of unknown compounds appeared in chick liver and kidney. No evidence was obtained for a significant accumulation of tocopheryl quinone or other metabolic products in either rat or chick tissues. α-Tocopherol in rat liver and intestinal mucosa cells was distributed about 50–60% in the mitochondria and 15–20% in the microsomal and supernatant fractions.

Relatively few detailed studies have been made on the metabolic fate of dietary α-tocopherol labeled with C^{14}. Johnson (1) reported briefly on the excretion by rats of injected labeled α-tocopheryl succinate, while Simon, Gross, and Milhorat (2) studied the absorption and excretion in rabbits. Sternberg and Pascoe-Dawson (3) investigated the distribution of radioactivity in tissues after administering α-tocopherol-C^{14}; however, no attempt was made to characterize the radioactivity, and the brevity of experimental details made interpretation of the data difficult. Martius and Costeli (4) found a radioactive quinone, which was not tocopheryl quinone, in liver of rabbits following the administration of α-tocopherol-C^{14}. Recently, several metabolites of α-tocopherol including α-tocopheryl quinone have been reported to be present in rat or pig tissues (5, 6, 7). With the advent of chromatographic procedures (8, 9), which permit the separation of α-tocopherol from most of the nonsaponifiable constituents in animal tissues, it was decided to reinvestigate the fate of this compound in the rat and the chicken.

METHODS

Crystalline d,α-tocopheryl-5-methyl-C^{14} succinate (specific activity 1.1 μc/mg)[1] was reduced with $LiAlH_4$ in diethyl ether (10), and the free tocopherol was subsequently purified by preparative chromatography on zinc carbonate-treated paper (8).[2] The purity of this preparation was established by column chromatography on a semimicro column of alumina and zinc carbonate (9). For administration to the experimental animals, the purified radioactive α-tocopherol was dispersed in water with 5% Tween 80 (polyoxyethylene sorbitan monooleate).

Weanling rats of the Sprague-Dawley strain were reared on a tocopherol-free diet containing 20% vitamin-free casein, 4% lard (stripped free of nonsaponifiable material by molecular distillation), 4% salt mixture, 72% sucrose, and all vitamins except E. The animals were used after three to four weeks when body weights were 120–180 g. Day-old, female chicks were fed a vitamin E-free diet containing 30% isolated soybean protein,[3] 4% stripped lard, 6% salts, 60% glucose, and all vitamins except E. In addition, 0.5 ppm selenium (as sodium selenite) was added to prevent exudative due to vitamin E and selenium deficiency. The chicks were used after four weeks. Under these conditions, the rats and chicks had very little α-tocopherol remaining in their livers (0.5 μg/g by chemical analysis [9]).

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[1] Distillation Products Industries, Rochester, N. Y.

[2] One batch was found to contain about 10% of γ-tocopherol, apparently a result of incomplete methylation in the synthetic process. Only the α-tocopherol homologue was used for experimentation.

[3] Archer, Daniel-Midland isolated soy protein C-1, Cincinnati, Ohio.

TABLE I. DISTRIBUTION OF RADIOACTIVITY IN THE TISSUES OF RATS AFTER A SINGLE ORAL DOSE OF 500 μg OF α-TOCOPHEROL-C^{14} (500,000 CPM)*

Tissue	% of Administered Dose in Whole Organ or Tissue		
	Hours After Dose		
	2.5	4.5	24†
Liver	6.40	5.40	5.53
Heart	0.06	0.06	0.37
Plasma‡	1.12	2.40	1.70
Erythrocytes	0.40	0.34	1.56
Small intestine	11.00	8.00	4.06
Kidneys	0.57	1.50	0.90
Brain	0.08	0.05	0.20
Spleen	0.11	0.14	1.16
Stomach	0.59	0.15	0.41
Lungs	0.15	0.32	0.84
Mesenteric fat (per g)§	0.12	0.31	0.88
Intestinal and stomach contents	27.0	8.12	1.05
Cecal contents and feces	6.80	28.20	39.82
Total	54.40	54.99	58.48

* Values are averages of results in two rats.

† Rats fitted with tail cups to prevent coprophagy.

‡ Total value calculated on the assumption that the rat contains 6% of its weight as blood.

§ This value expressed as percentage of dose per gram of tissue since no estimate of total mass of mesenteric fat was made.

Although partially depleted of their more labile stores of α-tocopherol, the animals were normal with respect to growth rate and appearance. By partially depleting the animals, it was hoped that the deposition of single doses of tocopherol would be enhanced compared with nondepleted animals.

The rats and chickens were fasted 24 hr and were given 0.5 ml of the α-tocopherol-C^{14} dispersion containing 500 μg (1 μg = 1,000 cpm) directly into the stomach (crop in the case of chicks) by a blunt needle and syringe. In some experiments, tail cups (11) were attached to the rats to prevent coprophagy. After the required time periods, the rats were anesthetized with light ether and killed by taking as much blood as possible from the heart with a heparinized syringe. Chickens were bled by heart puncture and subsequently killed by decapitation. Urine and feces of rats were separately collected using a metabolism cage, and the excreta of chickens were collected on polyethylene sheets. Tissues were removed immediately after death, weighed, and frozen at −20°. Intestinal and stomach contents were combined.

Isolation of the α-tocopherol from tissues and excreta prior to counting was performed by column chromatography after saponification and extraction as described previously (9). Urine samples were first hydrolyzed with 3 N HCl before extraction to free the tocopherol or possible metabolites from glucuronides (2). The feces, together with the colon and cecum and their contents, were homogenized in water in a blendor prior to saponification. Redistilled hexane was used in all extractions. The columns, containing a 1:1 mixture of alumina and zinc carbonate, were standardized with α-tocopherol, and only those eluate fractions corresponding to α-tocopherol were used for counting (α-tocopheryl quinone is retained on the column). Aliquots (10–100 μl) of the combined eluate fractions were spotted on a lens tissue in planchets (12) and spread uniformly to infinite thinness with the aid of 40 μl of 4% Tween 80 in ethanol. Radioactivity was measured in a windowless gas flow counter; a minimum of 1,000 counts was recorded except for very weak samples when 200 counts was the minimum.

Verification that the material in the column eluates that was counted was indeed α-tocopherol was made by chromatographing an aliquot of the pooled fractions on zinc carbonate-impregnated paper (8). The paper strips were sprayed with ferric chloride–bipyridyl reagent and then scanned with a radiochromatogram scanner. In all cases, only a single radioactive peak appeared corresponding to the colored zone with an R_F (0.4–0.5) identical to that of α-tocopherol.

RESULTS

The radioactivity in the nonsaponifiable extracts was accounted for almost entirely in eluate fractions 2 to 4 corresponding to α-tocopherol. In most tissue extracts, however, a small but significant amount (about 3–7%) of the radioactivity usually stayed at the origin of the paper or at the top of the chromatographic column and could be eluted with 95% ethanol. An aliquot of α-tocopherol-C^{14}, carried through the usual procedures of saponification, extraction, and chromatography, gave the same pattern as the tissue extracts, i.e., about 5% of the activity was very firmly adsorbed. It would appear, therefore, that some oxidation or degradation product of α-tocopherol always resulted from the process of saponification and extraction. Consequently, the presence of this small amount of non-α-tocopherol activity was considered to be of little significance.

The amount of radioactivity as α-tocopherol in the various tissues of rats killed 2.5, 4.5, and 24 hrs after a single oral dose of radioactive tocopherol is given in Table 1. α-Tocopherol-C^{14} appeared in all tissues after 2.5 hr, with liver and intestine having the most activity. The liver maintained a constant amount of activity from 2.5 to 24 hr, indicating that a steady state had been reached even within the first few hours.

TABLE 2. RECOVERY OF RADIOACTIVITY FROM THE RAT 24 HR AFTER A SINGLE ORAL DOSE OF 137 μg (137,000 CPM) OF α-TOCOPHEROL.*

Tissue or Excreta†	Total Activity	% of Dose
	cpm	
Liver	10,500	2.4
Organs‡	5,000	1.1
Intestinal tract + contents	203,150	46.5
Carcass	110,000	25.2
Feces	57,400	13.1
Urine, ether-soluble	360	0.1
Urine, glucuronide	1,840	0.4
Respiratory CO_2	2,880	0.7
	391,130	89.5

* Rat fasted 4 hr prior to dosing, then allowed food for remaining 24-hr period.

† Liver, organs, and intestinal tract with contents were ground with anhydrous sodium sulfate and extracted with acetone for 3 hr by mechanical shaking. Carcass was autoclaved and homogenized in a blendor with water, and an aliquot was extracted with chloroform–methanol 2:1. Feces were extracted with a mixture of $CHCl_3$, MeOH, and acetic acid. Urine was extracted with diethyl ether, then hydrolyzed with HCl and reextracted with ether. Respiratory CO_2 was collected in 5 N KOH and precipitated as $BaCO_3$.

‡ Kidneys, testes, lungs, heart, thymus, and spleen.

Heart, brain, spleen, and lungs had their highest value at 24 hr, while kidney appeared to reach a maximum at 4.5 hr. Stomach appeared to lose tocopherol by 4.5 hr, then regained it by 24 hr. The plasma activity showed a maximum at 4.5 hr, which decreased only slightly by 24 hr. The erythrocytes had a much smaller amount of radioactivity for the initial 4.5 hr and reached the same concentration as in plasma in 24 hr, indicating an equilibrium between the plasma and red cell. The uptake by the brain was slow compared to other tissues, in agreement with the recognized slow passage of lipids across the blood-brain barrier. The intestine had a considerable amount at 2.5 hr, which decreased gradually in 24 hr. The activity in mesenteric fat increased markedly during the 24-hr period. The total recovery in all of these tissues, and including the unabsorbed tocopherol, was 54–59% of the dose.

An effort to account quantitatively for the total radioactivity administered was made with one rat, 24 hr after dosing (Table 2). Radioactivity extracted from the samples in this experiment was not characterized; i.e., the extracts were not chromatographed but counted directly. The intestine, its contents, and feces accounted for 60% of the dose and 25% was present in the carcass. There was more in the liver (2.4%) than in all other organs combined (1.1%).

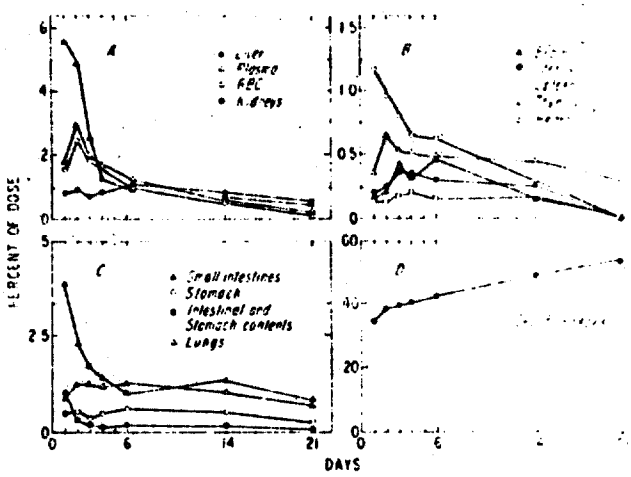

FIG. 1. Rate of depletion of α-tocopherol-C^{14} from rat tissues and the cumulative fecal excretion after a single oral dose of 500 μg. Points represent activity in the total organ or sample from two rats at each time interval.

Less than 1% was excreted in the respiratory gases and only 0.5% was in the urine, most of the latter activity being water-soluble.

In Fig. 1 are given the results of a longer time study of the rate of depletion of α-tocopherol-C^{14} after a single oral dose of 500 μg. Two animals were killed at the given time intervals and the mean value of the percentage radioactivity of the dose plotted for each tissue against time in days.

The uptake and depletion rate of α-tocopherol-C^{14} in the different tissues varied considerably. The plasma and erythrocytes followed a very similar pattern and by 21 days only negligible amounts of activity remained. In terms of absolute amounts, 3.6 μg of α-tocopherol was present in the total plasma and an almost equal amount in the erythrocytes. Although the initial uptake by liver was rapid, there was no appreciable retention. After six days, the amount of activity was only one-sixth that after one day. During the following two weeks, a slow rate of depletion occurred. The heart, kidneys, lungs, uterus, and thymus, however, maintained an almost constant amount for 14 days, then a gradual decrease followed. The curve for spleen resembled that for liver. It is interesting to note that both the small intestine and stomach retained a constant amount of tocopherol from the 14th to 21st days. The combined contents of the intestine and stomach had a small but definite amount of radioactive tocopherol at all time intervals. This activity in the intestinal tract was not due to recycling by coprophagy since the rats had tail cups. About 36–38% of the ingested dose was excreted within the first 24 hr in the feces, after which an almost constant amount of about 0.7–1% was excreted per day. The excreted radioactivity was completely accounted for as un-

TABLE 3. CONCENTRATION OF α-TOCOPHEROL-C^{14} IN RAT TISSUES AFTER AN ORAL DOSE OF 500 μg (500,000 CPM)*

Tissue	Days After Dose		
	1	14	21
	μg/g	μg/g	μg/g
Liver	7.4	0.6	0.4
Thymus	2.1	2.0	—
Stomach	3.3	3.5	2.1
Heart	3.4	4.6	3.0
Uterus	4.5	3.3	0.1
Kidneys	2.5	3.5	2.1
Lungs	4.6	5.0	4.4
Plasma (per ml)	3.2	0.7	0.1
Erythrocytes (per ml)	3.4	0.6	0.1
Small intestine	4.4	1.6	1.0

* Rats on experiment 14 and 21 days were fitted with tail cups to prevent coprophagy. Average of two rats at each period.

changed α-tocopherol, as determined by both paper and column chromatography. The radioactivity excreted in urine (not shown), which was essentially all water-soluble, was very low and reached a cumulative value of about 1% in 21 days.

Table 3 summarizes the amounts of α-tocopherol-C^{14} per gram of tissue 1, 14, and 21 days after dosage, as calculated from the radioactivity in the extracts. The concentration at the end of 21 days was highest in lungs, followed by heart, kidneys, and stomach. Liver, uterus, plasma, and red cells had very low concentrations, while the thymus had essentially none.

Intracellular Distribution of α-Tocopherol-C^{14} in the Liver and Intestinal Mucosa. An oral dose of 500 μg of α-tocopherol-C^{14} was given to two rats and after 6 hr the intestines and livers were removed. The intestinal mucosa was scraped off using a dull scalpel blade, and the mucosal cells and a piece of liver were homogenized separately in cold 0.25 M sucrose and fractionated by differential centrifugation (13). The cellular fractions and an aliquot of the whole homogenate were saponified, extracted, and counted after column chromatography. The results in Table 4 show that about 52 and 62% of the α-tocopherol is localized in the mitochondria of the liver and intestinal mucosal cells, respectively, while an appreciable portion is present in the microsomes and supernatant solution as well. Paper chromatography and radioactive scanning showed that all the fractions had only α-tocopherol.

Possible Metabolites in Liver. Two rats were each given 500,000 cpm of α-tocopherol-C^{14} by stomach tube and killed after 24 and 96 hr. One gram of liver was ground with anhydrous sodium sulfate and extracted with diethyl ether (fraction A). Another 1-g portion was homogenized in water and extracted with hexane (fraction B). To the aqueous layer was added an equal volume of 95% ethanol and the mixture reextracted with hexane (fraction C). The remaining aqueous layer was centrifuged to remove protein, and the clear supernatant solution (fraction D) was plated for counting. The radioactivity of each fraction, as well as the activity from the nonsaponifiable fraction of 1 g of liver, is shown in Table 5.

TABLE 4. INTRACELLULAR DISTRIBUTION OF α-TOCOPHEROL-C^{14} IN RAT LIVER AND INTESTINAL MUCOSA*

Fraction	Liver		Mucosa	
	cpm/g	%	cpm/g	%
Homogenate	8,800	100	20,388	100
Nuclei	571	6.5	1,800	8.8
Mitochondria	4,600	52.2	12,600	61.9
Microsomes	2,000	22.7	4,200	20.6
Supernatant solution	1,640	18.6	3,100	15.2

* 500 μg (500,00 cpm) α-tocopherol-C^{14} was given orally and the animals killed after 6 hr. Average of two rats.

Hexane alone extracted very little of the α-tocopherol from a water homogenate (fraction B), but, after denaturation of the protein with ethanol, 92–95% of the activity was fat-soluble (fraction C), indicating that the tocopherol is associated with the proteins. The aqueous layer remaining after extraction with ethanol-hexane contained about 1.5% of the total radioactivity, indicating that the amount of possible water-soluble metabolites is very small. The nonsaponifiable extract contained essentially the same amount of radioactivity as was obtained in the total lipid extraction with ether after grinding the tissue with sodium sulfate. Paper chromatography of the

TABLE 5. DISTRIBUTION OF RADIOACTIVITY FROM α-TOCOPHEROL-C^{14} IN RAT LIVER IN ETHER, HEXANE, AND WATER-SOLUBLE FRACTIONS*

Fraction	After 24 hr		After 96 hr	
	cpm/g	μg/g†	cpm/g	μg/g
A: Ether-soluble, from homogenate	7,250	—	3,960	—
B: Hexane-soluble, from homogenate	750	—	520	—
C: Hexane-soluble, after alcohol denaturation	6,830	—	3,630	—
D: Aqueous supernatant solution from C	120	—	60	—
Nonsaponifiable fraction	7,180	6.8	4,018	40

* See text for preparation of fractions. Male rats, depleted of vitamin E, were given 500,000 cpm (500 μg) of α-tocopherol-C^{14} orally and killed at intervals noted; one rat at each time.

† Determined colorimetrically.

TABLE 6. RADIOACTIVITY IN TISSUES OF CHICKS AFTER A SINGLE ORAL DOSE OF 500 μg OF α-TOCOPHEROL-C^{14} (500,000 CPM)*

Tissue	% of Administered Dose in Whole Tissue			Concentration of α-Tocopherol†		
	Day After Dose			Days After Dose		
	1	7	24	1	7	24
				μg/g		
Liver	1.90	0.52	0.10	0.70	0.40	0.05
Kidneys	0.20	0.14	0.04	0.50	0.37	0.02
Spleen	0.24	0.06	0.02	1.30	0.26	0.11
Heart	0.26	0.08	0.03	0.60	0.22	0.08
Plasma‡	4.00	1.92	0.03	1.00	0.50	0.07
Erythrocytes‡	3.20	1.20	0.03	0.86	0.34	0.08
Leg muscle	—	—	—	0.13	0.32	0.16
Abdominal fat	—	—	—	—	—	0.35
Excreta§	15.1	18.1	22.5	—	—	—

* Average values of two chicks at each period. Weights ranged from 300-400 g when dosed. Blanks indicate no estimation was made.

† Calculated from the radioactivity.

‡ Blood volume estimated as 10% of body weight.

§ Cumulative value for the entire period.

ether and hexane extracts indicated a single radioactive zone corresponding in R_F to α-tocopherol.

Chick Experiments. The distribution of radioactivity in some of the tissues of the chicken at intervals after a single oral dose of α-tocopherol-C^{14} is summarized in Table 6. Blood and liver had the greatest amount of total activity after one and seven days, but the rate of loss was remarkably similar in most tissues. Kidney appeared to have a relatively constant amount from days one to seven, while leg muscle accumulated tocopherol during this period. Of particular interest was the apparent rapid equilibrium between plasma and erythrocytes, which persisted even after one week. In terms of concentration, spleen was highest after one day followed by the blood, liver, kidneys, and heart. By seven days, the range of concentrations in all tissues was only about twofold. Very little activity remained in any tissue after 24 days; however, abdominal fat had a significant concentration.

The data for the chicks killed after one day show that the tissues analyzed accounted for only about 10% of the dose while 15% was recovered in the excreta. In view of the difficulties in collecting chick excreta quantitatively, it is probable that this latter figure is low. Paper chromatography revealed that only α-tocopherol was present in the extracts of excreta.

The radioactivity of all tissues obtained on the first and seventh days was due entirely to unchanged α-tocopherol as shown by counting the various fractions eluted from the alumina-zinc carbonate column. However, the 24th-day tissue samples (liver, kidneys, and plasmas) on column chromatography (Table 7) showed that the major fraction of radioactivity was not due to unchanged α-tocopherol, although the leg muscle and erythrocytes still contained most of their radioactivity as α-tocopherol. The activity in fraction 8 could be α-tocopheryl quinone since, with this column, the quinone is eluted with ethanol. The nature of the activity in fraction 6 is unknown.

In Vitro Incubation of α-Tocopherol-C^{14} with a Liver Extract. A 10% homogenate of liver from a vitamin E-depleted chick was prepared in 0.1 M phosphate buffer at pH 7.4 and centrifuged at 800 × *g* for 10 min. To 2 ml of the supernatant solution were added 3.25 ml of buffer, 0.5 ml of an emulsion of 80 μg of α-tocopherol-C^{14} dispersed in 5% Tween 80, and 0.25 ml of an emulsion of 500 μg ethoxyquin[4] in water as antioxidant. The flask was incubated in air at 37° for 3 hr. At 60-min intervals, 1 ml was removed, the protein precipitated with ethanol, and the mixture extracted with diethyl ether. The extracts were chromatographed on the column of alumina and zinc carbonate, and aliquots of the eluate fractions were counted. Other aliquots were chromatographed on zinc carbonate paper and subsequently scanned. More than 95% of the starting activity was recovered at each time interval, and all of the activity was accounted for as α-tocopherol. Under these conditions, the liver does not metabolize or degrade α-tocopherol.

TABLE 7. DISTRIBUTION OF RADIOACTIVITY IN CHROMATOGRAPHIC FRACTIONS OF NONSAPONIFIABLE MATTER FROM CHICK ORGANS AND TISSUES 24 DAYS AFTER A SINGLE DOSE OF 500 μG (500,000 CPM) OF α-TOCOPHEROL-C^{14}*

Eluate Fraction	Liver	Kidneys	Plasma	Erythrocytes	Leg Muscle
	cpm per g or ml				
1	1	0	0	0	6
2, 3	8	16	7	59	89
4	0	4	2	12	14
5	0	0	0	0	0
6	22	120	67	0	0
7	0	0	5	0	0
8	23	22	45	16	29
% Recovery†	108	105	111	105	98

* The column of alumina and zinc carbonate (8) was used and 1-ml fractions collected. Fractions 2, 3, and 4 represent unchanged α-tocopherol. Fractions 1-7 were eluted with 1 ml of 15% benzene in hexane, fraction 8 with 2 ml of 95% ethanol.

† Recovery calculated on the original counts of the samples.

[4] 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline ("Santoquin"), Monsanto Chemical Co., St. Louis, Missouri.

DISCUSSION

By using the oral route of administration and with a dose level approximating a reasonable dietary intake, the present experiments should reflect fairly accurately the normal distribution and fate of ingested α-tocopherol.

It is apparent from the experiments with both rats and chicks that different tissues of the body vary considerably in their rate of uptake and depletion. Although liver and blood accounted for most of the dose during the first several days, these tissues rapidly lost α-tocopherol and after three weeks the concentration was less than 1 μg/g (or milliliter). In contrast, several organs in the rat that do not build up relatively high concentrations appear to retain a constant amount for at least three weeks. Thus, α-tocopherol in lung, kidney, heart, and stomach had a slow turnover rate and significant concentrations (2–4 μg/g) persisted at 21 days. This suggests that these organs would be better criteria of the tocopherol status of an animal, from the biochemical viewpoint, than would blood or liver. The latter tissues, however, would reflect current nutritional status. Thymus and uterus, like liver, had concentrations of less than 1 μg/g after three weeks.

Several recent reports have appeared indicating the formation of various metabolites in the rat from α-tocopherol. Csallany et al. (7) have isolated tocopheryl quinone following the injection of d,α-tocopherol-C^{14}, and, in another communication (6), the same group reported a second oxidation product of α-tocopherol in rat liver. Simon et al. (2) showed that rabbits can oxidize the tocol portion of α-tocopherol to a quinone (tocopheronolactone), which is excreted in the urine as a glucuronide. We found barely detectable evidence for this compound in rats. The excretion of radioactivity in urine in our rat experiments was about 1% of the total dose in 21 days. Sternberg and Pascoe-Dawson (3) reported a considerable excretion (37%) via urine in 64 hr. However, our values are in agreement with the results of Johnson (1), who reported less than 1% excretion via urine in the first 48 hr in the rat.

Inasmuch as we found no tocopheryl quinone or the other oxidation product described by Draper and co-workers (6, 7) after *oral* administration of α-tocopherol-C^{14} in rats, it would appear that the quinone is a negligible metabolite by the normal pathway. Morton and Phillips (14) have found small amounts of tocopheryl quinone in some, but apparently not all, livers from normal rats. As pointed out above, a compound amounting to about 5% of the total activity is invariably present as an artifact in all tissue extracts carried through the procedures of saponification and extraction.

The activity due to α-tocopherol, which Csallany et al. (7) isolated from rat liver two days after injection of α-tocopherol-C^{14}, was only 25% of the total radioactivity. In contrast, we invariably found over 90% of the activity in the liver to be due to unchanged α-tocopherol after *oral* dosing, regardless of the time interval.

The distribution of α-tocopherol in rat liver cellular fractions confirms previous reports that vitamin E, as also vitamin A (15) and ubiquinone (16), is not confined to any one compartment. The amount we found in rat liver mitochondria, 52%, is about twice that in chicken liver mitochondria (14). No evidence was found for the presence in mitochondria of a quinone derived from α-tocopherol-C^{14} and suggested by Martius and Costeli (4) to be phyllobenzoquinone.

The results with chickens are in general agreement with those obtained for rats. The amounts deposited in tissues were, however, much lower, in accordance with the lower dosage as related to body weight. Twenty-four days after the administration of radioactive α-tocopherol, all the tissues were practically depleted of the compound; the only exception was abdominal fat, which still contained 0.25 μg g. The small amount of activity (100 cpm/g) present in most tissues at this time was found by chromatography on the alumina-zinc carbonate column to contain one or two nontocopherol compounds. As described in Table 7, the consistent recovery of a marked amount of radioactivity in fraction 6 from several tissues (liver, kidney, and plasma) indicates the presence of a degradation product or a possible metabolite in these tissues. On the other hand, all the tissue nonsaponifiable fractions contained a strongly absorbing substance, elutable with ethanol (fraction 8), at a much higher percentage than was obtained with similar tissue extracts one and seven days after dosing. The chromatographic behavior of fraction 8 suggests that it could be tocopheryl quinone. Neither fraction 6 nor 8 could contain the dimer of α-tocopherol obtained by potassium ferricyanide oxidation (6, 17, 18). An authentic sample of this dimer, prepared by Dr. W. Durckheimer and L. A. Cohen in the Laboratory of Chemistry of this Institute, was found to be eluted in fractions 1 to 3. The dimer is nonreducing and has an R_F on the zinc carbonate paper considerably greater than that of α-tocopherol (0.95 vs 0.50). Thus, the dimer would not have been included in the colorimetric determination of α-tocopherol, nor would it have been overlooked in the radioactive scanning of the paper strips.

The absence of metabolites of α-tocopherol in the tissues of our rats and chicks (except for 24-day-old chicks) is at variance with the results of several investigators as noted above. Possible reasons for these discrepancies have already been indicated; namely, the intraperitoneal route may provide oxidative reactions that are either greatly diminished or absent when tocopherol is absorbed via the intestine. In addition, it is very probable that large, oral doses are partially shunted into pathways not used for smaller doses. This may be the explanation for the formation of tocopheronolactone; however, a species difference cannot be ruled out. A further variable might be the possible effect of tocopherol status; i.e., chronically deficient, depleted, or normal animals. Although there can be no question that α-tocopherol is degraded in the body, our study indicates that the molecule is relatively stable, and that, once degradative attack begins, there is not significant accumulation of intermediate products.

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5[illegible]; Toxic effect of excessive doses of vitamin A and of vitamin A plus tocopherol on oxidative phosphorylation in rat liver mitochondria. Kryukova, L. V.; Pechenina, M. I. (Altai. Med. Inst., Barnaul, USSR). *Farmakol. Toksikol.* 1968, 31(6), [illegible] (Russ.). Vitamin A administered at [illegible] or 20,000 I.U. with the food to rats daily for [illegible] days decreased the oxidative phosphorylation coefficient in the mitochondria. Tocopherol (vitamin E) (50 mg.) administered against a background of 20,000 I.U. of vitamin A did not normalize the P/O ratio, but disrupted it even further, apparently due to mitochondrial damage. BJJR

ON THE TOXIC EFFECT OF EXCESSIVE DOSES OF VITAMIN A AND ITS COMBINATION WITH TOCOPHEROL ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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The purpose of our research was the study of oxidative phosphorylation in the liver during the introduction of both large and fully permissible amounts of vitamin A into the organism, exceeding the daily requirement insignificantly. Moreover, the problem concerning the effect of the combined use of vitamins A and E on the process of tissue respiration interested us. During A-hypervitaminosis, relative to the intensification of oxidative processes, the absorption of oxygen by the tissues is increased. A similar occurrence is also observed during E-vitaminosis (Houchine, Weinstock, and coauthors). In connection with this, it is natural to attempt to use vitamin E during A-hypervitaminosis for the normalization of disrupted oxidative processes. Tocopherol, an antioxidant, protects vitamin A from deterioration and may, thereby, promote its accumulation in the organism.

Procedure

We conducted tests on four groups of rats weighing nearly 200 g. The first group (7 rats) was controlled; the second (10 rats) supplementarily received 20,000 I. U. (international units) of vitamin A daily per os in (their) food, in the form of an oil concentrate; the third (8 rats) - 20,000 I. U. of

vitamin A and 50 mg. of vitamin E; the fourth - 400 I. U. of vitamin A (6 rats). All animals remained on standard feeding. The duration of the tests for rats of the second and third groups amounted to nearly 50 days on the average; for rats of the fourth group - nearly 90 days. The animals were killed by decapitation. Nearly 2 g. of liver was immediately extracted and placed in a 0.25 M (molarity) solution of saccharose, chilled until the appearance of ice. (The liver) was quickly cut into little pieces with scissors in the cold and homogenized for 20 seconds in a glass homogenizer in a 10-multiple volume 0.25 M saccharose solution. (The pieces were then) centrifuged for 5 minutes at 0 degrees and a gravity of 60. (This) super-sedimentary fluid was centrifuged once more at 0 degrees and a gravity of 8000 for a period of 10 minutes. The mitochondria sedimentation was scrubbed twice with a 0.25 M saccharose solution, each time separated by its centrifugation at a gravity of 8000 in the cold. The mitochondria were suspended in a 0.25 M saccharose solution with such sizing that their volume, corresponding to 0.35 g. (350 mg.) of liver, was contained in 0.3 ml. of the suspended matter. The absorption of oxygen and loss of inorganic phosphorous was determined on a Barburg-type instrument. A written example of the incubative mixture is taken from the work of N. A. Glotov. Incubation was conducted for 30 minutes in an air atmosphere at 26 degrees. The intensity of phosphorylation was seen (to be) in accordance with the loss of inorganic phosphorous. The absorption of oxygen was determined manometrically. The fermentative process in the vasculum was terminated with the addition of 2 ml. of a 5% solution of trichloroacetic acid.

Results

In animals of the second group, the expenditure of inorganic phosphorous, in comparison with the norm, was reduced; during the consumption of alpha-ketoglutarate, to 5.7 $\pm$ 0.33 micromole (P=0.01); during the consumption of succinate, to

4.9 ± 0.36 micromole ($P<0.02$). The expenditure of oxygen, on the other hand, increased: in tests with alpha-ketoglutarate, to 6.7 ± 0.4 micromole ($P<0.01$); in tests with succinate, to 9.8 ± 0.11 micromole ($P=0.01$). This led to a significant decrease in the size of the P/O ratio for alpha-ketoglutarate, to 0.85 ± 0.08 micromole ($P<0.001$), which is below the norm by 47%; for succinate - to 0.055 micromole ($P<0.001$) - below the norm by 58%.

Tests on rats of the third group showed the following. The amount of inorganic phosphorous loss did not differ from that observed in the second group of animals, receiving only vitamin A. The intensity of (tissue) respiration during the consumption of alpha-ketoglutarate also did not differ from the corresponding indicator in the second group, and, therefore, exceeded the norm. Regarding tests with succinate, in this instance, the expenditure of oxygen was much less than in rats of the second group ($P<0.001$), and approached the norm. The P/O coefficients for both substrata were significantly below the norm and neared the indices of the second group.

In rats of the fourth group, receiving 400 I. U. of vitamin A: in tests with alpha-ketoglutarate, a significant decrease in the expenditure of inorganic phosphorous was noted as compared to the norm ($P<0.01$); in tests with succinate, it did not differ from the norm. An increase in the expenditure of oxygen was statistically uncertain for both substrata; however, the size of the oxidative phosphorylation P/O coefficient was clearly decreased. For alpha-ketoglutarate, this decrease amounted to 47% ($P<0.01$); for succinate - 41% ($P<0.02$).

Thus, summing up the obtained material, the following may be concluded. The introduction of a dose of vitamin A into rats over the course of 50 days, exceeding the daily requirement a thousandfold, led to a decrease in the consumption of inorganic phosphorous liver mitochondria, and to a compensatory increase in the absorption of oxygen. This entailed a sharp

decrease in the P/O coefficient, appearing as an indicator of the intensity of oxidative phosphorylation. The use of tocopherol against a background introduction of a large dose of vitamin A decreased the expenditure of oxygen during the use of succinate as a respiration substratum. But, inasmuch as this effect was not accompanied by an increase in the consumption of mineral phosphorous mitochondria, it is necessary to assess the development of a more profound impairment of the mitochondrium structure in (this test), than in tests with the use of only vitamin A. It is possible that vitamin E, being an antioxidant, inhibited the breakdown of vitamin A in the organism and thereby promoted its more prolonged and profound effect on the mitochondrium.

In our opinion, tests on rats receiving 400 I. U. of vitamin A over the course of nearly 90 days have significant interest. In this case, the expenditure of oxygen was changed insignificantly, but the consumption of inorganic phosphorous was decreased, which led to a significant decrease in the effectiveness of oxidative phosphorylation. The prolonged application of such a dosage apparently led to a breakdown of the mitochondrium structure.

The obtained data point to the need for comprehensive research on the acceptability of the use of this or other dosages of vitamin A, and also of its combination with vitamin E, in each specific case.

Conclusions

1. The introduction of 400 I. U. of vitamin A daily into rats per os decreases the oxidative phosphorylation P/O coefficient in rat liver mitochondria in the same way as the introduction of 20,000 I. U.

2. The application of 50 mg. of tocopherol daily to a rat, together with 20,000 I. U. of vitamin A, does not normalize the process of oxidative phosphorylation, but, on the contrary, promotes their still greater breakdown.

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TOXIC ACTION OF EXCESSIVE VITAMIN A DOSES AND OF ITS COMBINATION WITH TOCOPHEROL ON THE OXIDATIVE PHOSPHORILATION IN MITOCHONDRIA OF THE RAT'S LIVER

L. V. Kryukova, M. I. Pechenina

Albino rats were given vitamin A in an amount of 400 I. E. and 20000 I. E. per day for 90—95 successive days. A fall of the oxidative phosphorilation ratio P/O in mitochondia of the rat's liver was registered. Administration of 50 mg of α-tocopherol together with 20000 I. E. of vitamin A failed to normalize this ratio.

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Influence of Alpha-Tocopherol upon Development of Cardiovascular Necrosis and Hypertension in the Rat.* (20970)

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Medical literature is replete with contradictory reports on therapeutic efficacy of vit. E. Thus large dosages of alpha-tocopherol or mixed tocopherols have been reported to exert therapeutic effects in muscular dystrophies and rheumatic diseases of men(1-3). Also that prolonged administration of this vitamin may bring about dramatic amelioration in hypertensive cardiovascular disease, as well as many peripheral vascular conditions(4-6). However, well controlled clinical studies by others failed to confirm the reported benefits(7-10). The vast animal experimental literature dealing with vit. E contains surprisingly few references to the beneficial action of this vitamin, if one takes exception to specific effects in induced deficiency. With regard to the cardiovascular system, only 2 publications described therapeutically desirable effects. Allardyce and associates(11) reported a hypotensive effect of vit. E in desoxycorticosterone induced hypertension of albino rats, and Holman(12) was able with the help of vit. E to inhibit emergence of experimental necrotizing ar-

* This investigation aided by a grant from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

teritis induced in dogs on a "standard fat" diet by way of renal injury.

In view of these latter reports and the controversial nature of clinical observations, it seemed of interest to evaluate the influence of vit. E upon development of hypertension and cardiovascular necrosis induced in the albino rat with the help of renal injury, using a short-term method developed in our laboratory(13).

Materials and methods. Closely bred 10-months-old albino rats of both sexes from our own colony were employed. Animals were maintained on Purina Chow. For the actual tests, subgroups of at least 5 rats each were placed on a special complete synthetic diet (14). Since cardiovascular necrosis and hypertension were produced by procedure previously described(13), only the main features of this method will be briefly restated. A single excessive dose of sodium acetyl sulfathiazole is injected intraperitoneally into adult albino rats. This induces severe "obstructive nephropathy" due to massive deposition of poorly soluble sulfonamide crystals in the renal tubules. Impairment of the kidney in turn results almost invariably in the development of extensive disseminated necrosis in the arterial tree as well as the myocardium, and frequently also, in the muscularis of the stomach within a period of 5 to 7 days. Necrosis is followed by a stage of reparative changes with scar formation and intense calcification. Marked hypertension developing about 5 days after renal block, lasting for 2 to 5 weeks, is a usual feature of the intoxication. It was calculated that control animals eating standard synthetic diet received about 0.8 mg of alpha-tocopherol daily. Experimental diet was enriched to yield a daily intake of 10 mg alpha-tocopherol† per rat. After 2 weeks, rats eating the enriched food received supplementary stomach tube feedings of alpha-tocopherol in olive oil (10 mg/100 g body weight), 3 times weekly for 4 weeks. This was to ensure an uninterrupted intake of alpha-tocopherol in excess, since spontaneous food consumption drops sharply after production of renal injury. Rats eating the normal synthetic diet received intubations of equal volumes of olive oil. Kidney damage was induced in both groups after third intubation. A total of 33 rats were employed. Of these, 2 groups of 5 animals each, served as normal diet and vit. E diet controls, respectively. They were not exposed to renal injury and were killed after completing the full course of alpha-tocopherol or olive oil intubation on the 29th day. In the experimental group exposed to renal injury and subsequent cardiovascular damage, 13 rats (8 ♂ and 5 ♀) were kept on alpha-tocopherol enriched regimen, whereas 10 rats (5 of each sex) were fed the control synthetic diet.

Food intake was determined daily and the body weight checked twice weekly. Systolic blood pressure was recorded at predetermined intervals with the help of a photoelectric tensometer which measures volume changes in the hind leg of unanesthetized rat(15). All measurements were made by one experienced experimenter. Except for an occasional moribund animal which was not subjected to the recording procedure, all control and experimental rats were measured at the same session. The mean of at least 3 to 5 consecutive satisfactory readings at intervals of several minutes was taken to represent the systolic blood pressure. The animals were killed at predetermined intervals by exsanguination in ether anesthesia. Blood was used for estimation of N.P.N., calcium, and inorganic phosphates. All rats, including those dying spontaneously as a consequence of renal injury, were subjected to complete postmortem examination. The heart, thymus gland, adrenals, and kidneys were weighed immediately after removal on a torsion balance to the nearest mg. These organs as well as the aorta and gastrointestinal tract, were examined under the microscope using routine and special stains.

Results. Data on the incidence and severity of the most important organic lesions found in experimental animals, after exposure to renal injury, are depicted in Table I and Fig. 1.

A glance at the life span of individual ani-

† Liberal supplies of alpha-tocopherol acetate were obtained through the courtesy of Dr. A. Pirk of Hoffmann-La Roche, Inc., Nutley, N. J.

TABLE I. Production of Disseminated Muscular Necrosis in the Albino Rat. Influence of excessive amounts of alpha-tocopherol.

Days killed or died* after renal block	Heart, necrosis, calcification, cell infiltr. Myocard.	Coron. vessels	Aorta, medio-necrosis, perivasc. infiltration	Stomach, necrosis and calcification, muscularis	Kidney, pyeloneph. calcification, nephrosis
Alpha-tocopherol enriched synthetic diet					
Males					
6*	3+	3+	3+	+	4+
8*	2+	+	4+	3+	4+
9*	3+	3+	4+	—	3+
13*	3+	2+	4+	0	4+
16*	2+	3+	4+	0	3+
29	—	3+	2+	±	3+
29	—	2+	4+	2+	3+
29	—	2+	4+	3+	3+
Females					
7*	2+	3+	4+	0	3+
13*	2+		4+	0	3+
23	+	2+	4+	+	3+
23*	3+	3+	4+	—	4+
50	±	±	2+	—	3+
Normal synthetic diet					
Males					
7*	3+	+	±	—	2+
7*	±	—	—	—	2+
8*	2+	+	±	—	3+
62	—		3+	—	3+
62	—	+	—	—	3+
Females					
7*	3+	2+	3+	0	4+
13*	4+	2+	3+	0	4+
62	—	+	+	—	+
63	—	2+	4+	+	3+
50	+	2+	3+	—	4+

— = Normal organ (macroscopically and microscopically).
± = Definite but minor damage, recognizable under microscope.
+ = Considerable damage, recognizable under microscope.
2+ = Marked change, often visible to naked eye.
3+ = Advanced change, easily recognized upon gross inspection (except coronary vessels).
4+ = Severest degree of abnormality.
0 = Organ not examined.

mals (Table I) demonstrates that the extent of vascular, gastric or renal changes is not noticeably altered by variations in time of survival from 6 to >60 days following renal block. Myocardial damage, on the other hand, is most conspicuous within the first 2 weeks and shows a tendency towards complete healing in animals surviving 4 weeks or longer. Accordingly all 14 rats that died within the first 23 days, showed myocardial damage, usually of marked degree, whereas of the remaining 9 animals surviving more than 4 weeks only 2 manifested minor changes in the heart muscle. In view of the fact that most of these latter animals still had severe renal and vascular damage, it was assumed that they probably also had myocardial changes at an earlier date. This is supported by our findings in other recent studies with groups of rats killed within 14 days after renal block. As in the corresponding group of the present study, the incidence of typical myocardial alterations was usually close to 100%. It is obvious, therefore, that in comparative evaluation of changes in the heart, time elapsing between renal injury and death is of major significance.

With regard to changes in the muscularis

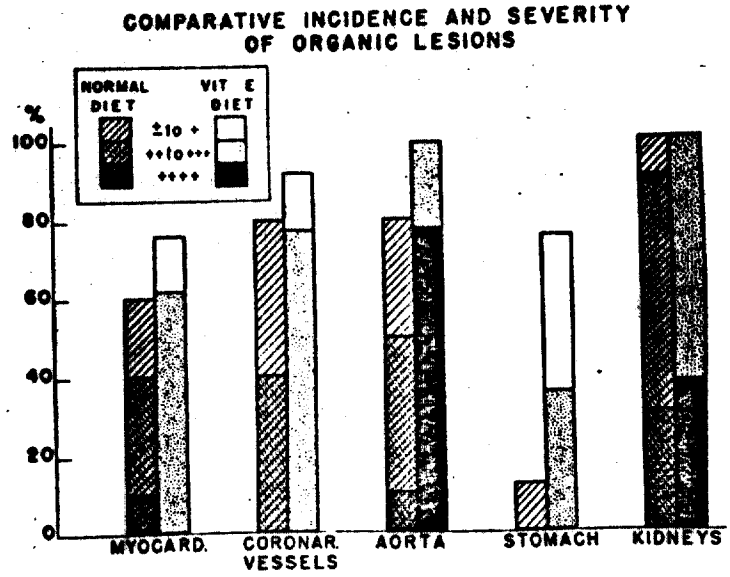

FIG. 1. Note substantial difference in *severity* of organic changes in coronary vessels, aorta and stomach of albino rats. (For explanation of + and — symbols see Table I.)

of the stomach it should be pointed out that advanced autolysis made it impossible to examine the stomachs of 6 rats. Of these, 2 were on control diet and 4 on vit. E enriched regimen. Nevertheless, the absence of any damage in the stomach wall of 7 out of 8 rats eating the normal diet, and the presence of necrosis in the muscularis of 6 out of 9 stomachs examined, from the vit. E group including 3 with advanced necrosis and calcification, adds considerable weight to the impression that damage of smooth muscles is enhanced in the presence of alpha-tocopherol in large amounts. This becomes particularly noticeable when the pathologic-anatomical alterations are grouped in accordance with the degree of damage (Fig. 1). Although, except for the stomach, total incidence of organic lesions is apparently not materially greater in rats receiving large dosages of alpha-tocopherol than in animals maintained on a normal diet, the distribution of coronary and especially aortic lesions according to extent of damage is highly suggestive of an aggravating effect of alpha-tocopherol.

Of the 5 control rats maintained on the alpha-tocopherol enriched regimen without being exposed to renal injury, 2 animals showed small foci of nephritis as the sole organic change. This insignificant lesion is not infrequently observed as a spontaneous change in normal rats.

No substantial deviations from the norm were found in the blood calcium and inorganic phosphate levels of experimental animals, as determined in samples obtained at sacrifice, which meant at least 3 to 4 weeks after date of renal injury. The N.P.N. levels, on the other hand, were still elevated in many instances and remained above the normal range even after 50 and 60 days.

Following production of renal block, the rats of both diet groups suffered usual loss of body weight, about 20% after one week and more than 40% in 3 weeks. Thereafter, with increasing appetite, body weight usually returned to its initial value at about the fifth week. Control animals on both diets showed expected maintenance or slight gain in body weight.

Finally, the effect of alpha-tocopherol upon the systolic blood pressure of normotensive and hypertensive albino rats is depicted in form of a scatter-gram (Fig. 2). The figure contains individual blood pressure records of all 23 experimental rats on 5th and 3rd day before production of renal block and of survivors of this group on the 8th, 16th, and 22nd day after renal injury. In addition, this graph also contains blood pressure read-

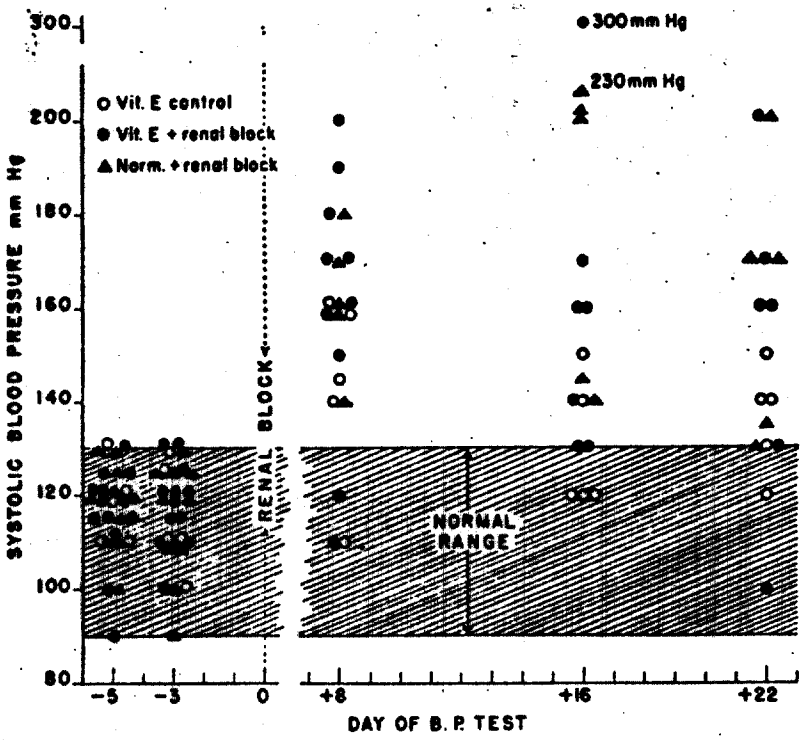

FIG. 2. Individual systolic blood pressures of 28 albino rats. In 23 of these rats a renal block was induced at zero day. The other 5 animals served as vit. E diet controls. Note marked hypertension resulting from renal injury and moderate elevation of systolic blood pressure caused by alpha-tocopherol administration.

ings of 5 control animals exposed to vit. E diet regimen. It is apparent that definite though moderately hypertensive levels were registered in some of the control rats on several occasions under the influence of alpha-tocopherol intubation (white circles). In contrast the blood pressure of 5 controls intubated with olive oil only, remained within normal range. (For the sake of greater clarity the blood pressure values of these latter controls were not included in the graph.)

Renal block, on the other hand, induced expected pronounced elevation of systolic blood pressure in the majority of experimental animals. No substantial difference in incidence or degree of hypertension could be derived from this small group of survivors feeding on either of the 2 diets. It is quite clear, therefore, that presence of abundant amounts of alpha-tocopherol in the body of the albino rat, does not noticeably interfere with the development of renal hypertension (black circles).

During the 14-day period of control observations before production of renal damage, systolic blood pressures of all experimental and control rats had kept well within normal range of 90 to 130 mm Hg.

Characteristic pathological anatomical changes in the cardiovascular system, gastrointestinal tract, adrenals, thymus gland, and kidneys were closely similar upon gross inspection and under the microscope to those observed in previous studies(14); also the change in moist weight of the 3 last named organs. The macroscopic and microscopic appearance of vascular lesions is illustrated in Fig. 3. Typical histological changes in the kidneys, the myocardium and the muscularis of the stomach are shown in Fig. 4. Lesions produced in presence of alpha-tocopherol in excess appeared to differ in degree of intensity rather than in quality from the organic damage induced in rats on a normal diet.

Comments. This study demonstrated that under the conditions of our experiments,

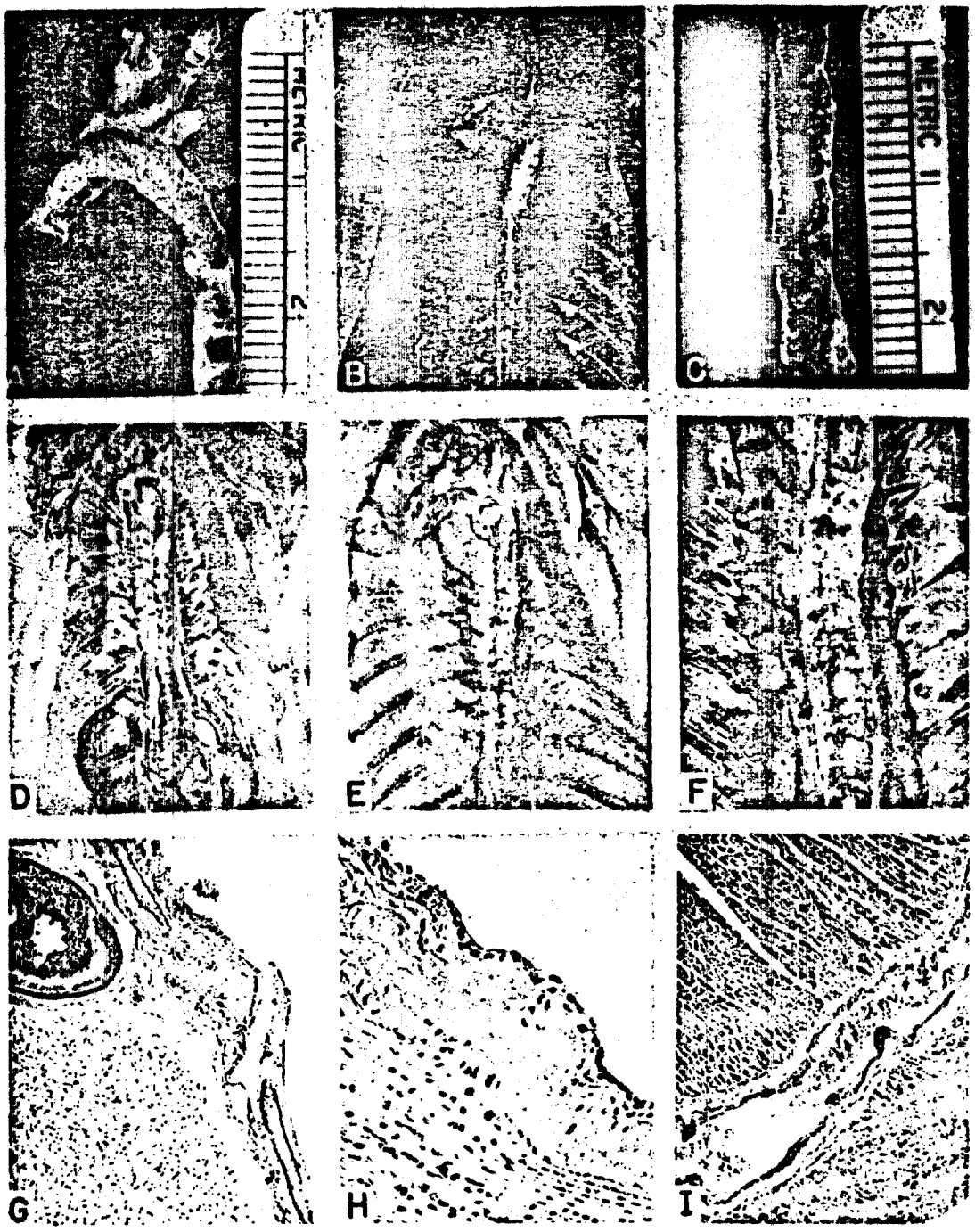

FIG. 3. Typical macroscopic and microscopic appearance of arterial lesions in alpha-tocopherol fed albino rats following renal injury. (Animals 1-13, Table I.)

A. Aortic arch and large branches. Bamboo-stick lesion of aorta extending into innominate, carotid and subclavian arteries.

B. Calcified aneurysm of aortic arch and upper part of descending aorta. Note normal thin-walled aorta below crossing of V. azygos. (Protection apparently due to mechanical pressure of vein.)

C. Abdominal aorta. Multiple aneurysmic dilatations of thinned out wall.

D. Dilation and bamboo-stick calcification of arch and descending aorta. Also visible are enlarged adrenal glands and contracted kidneys.

E. Open view of the aorta of Fig. D.

F. Same as Fig. E, close-up. Note irregular, bulging sub-intimal nodules. There is superficial resemblance to human atheromathosis.

G. Aorta and a large branch. (Left upper corner of photograph) showing necrosis and calcification of media.

H. Wall of aorta. Subintimal necrosis of media with swelling and breakdown of elastic membranes, and proliferation of endothelial cells over the lesion.

I. Longitudinal section of coronary artery. Wall is markedly thinned out, necrotic and irregularly calcified; lumen is filled with coagulated plasma and red cells.

alpha-tocopherol had no ameliorating influence upon the development of hypertension nor upon cardiovascular necrosis induced in the albino rat by way of renal injury. On the contrary, the presence of abundant supplies of this vitamin appeared to enhance muscular necrosis in the wall of blood vessels and stomach of these animals. In addition, alpha-tocopherol was found to be capable of producing moderate elevation of systolic blood pressure of normotensive control rats. These observations are at variance with the results of 2 experimental studies previously mentioned. Thus Allardyce and associates(11) reported that during daily administration of vit. E in 10 mg dosages (route of administration not stated), systolic blood pressure of rats, apparently stabilized at 300 mm Hg due to administration of desoxycorticosterone, showed a gradual decline to 250 mm Hg. However, only 6 rats were employed. Since only 3 of these animals received vitamin alone, whereas the remaining 3 were given histidine concomitantly, the significance of the observation is open to doubt. Our contrary finding of a blood pressure raising effect of vit. E is in line with the warning of Shute(16) that large dosages of alpha-tocopherol (300 mg or more daily) may cause a very considerable rise in both the systolic and the diastolic blood pressure of patients with hypertensive heart disease. It is also in conformity with the experimental data of Telford and associates (17) who reported about 30% reduction of systolic blood pressure in vit. E deficient rats as compared to normal control animals. Holman(12) who saw a preventive effect of vit. E in experimental arteritis induced in 8 dogs by "standard fat diet" and "standard renal damage," suggested that arterial lesions are induced by piling up of "toxic" substances (fatty acids) which are prevented from spilling over into urine when the safety valve of the kidney is destroyed.

The vascular lesions which we observed in albino rats were similar though far more severe than those seen by Holman. And yet, they developed in the absence of cholesterol and all animal fats from the diet of this species. Moreover, in experimental atherosclerosis caused by cholesterol feeding, vit. E was reported to have either no beneficial action(18) or to be responsible for accentuation of the characteristic arterial changes(19-21).

In trying to explain beneficial or detrimental effects of tocopherols, one becomes keenly aware that, among vitamins, vit. E in the physiology of the body is least well understood. It has been suggested, on the basis of knowledge of the effects of vit. E deficiency in the rat, that a major role of the vitamin *in vivo* is the control or restraint of one or more oxidative reactions, either by limiting the rate of some normally occurring reactions or by preventing the occurrence of detrimental reactions that take place only in its absence (22). It is well known that deprivation of this vitamin is followed by a bewildering array of functional and organic disturbances in different species of animals and that many of these disturbances can be cured in a striking manner by alpha-tocopherol administration (23). There should, of course, be no reason to assume that presence of large amounts of a substance must necessarily have a beneficial action upon tissues which suffer damage due to its depletion, and yet, judging from clinical reports, much of the reasoning in therapeutic application of vit. E seems to have proceeded in this manner.

In view of the many contradictory claims regarding the therapeutic capabilities of vit. E at the bedside, it would seem desirable that a more determined effort be made to distin-

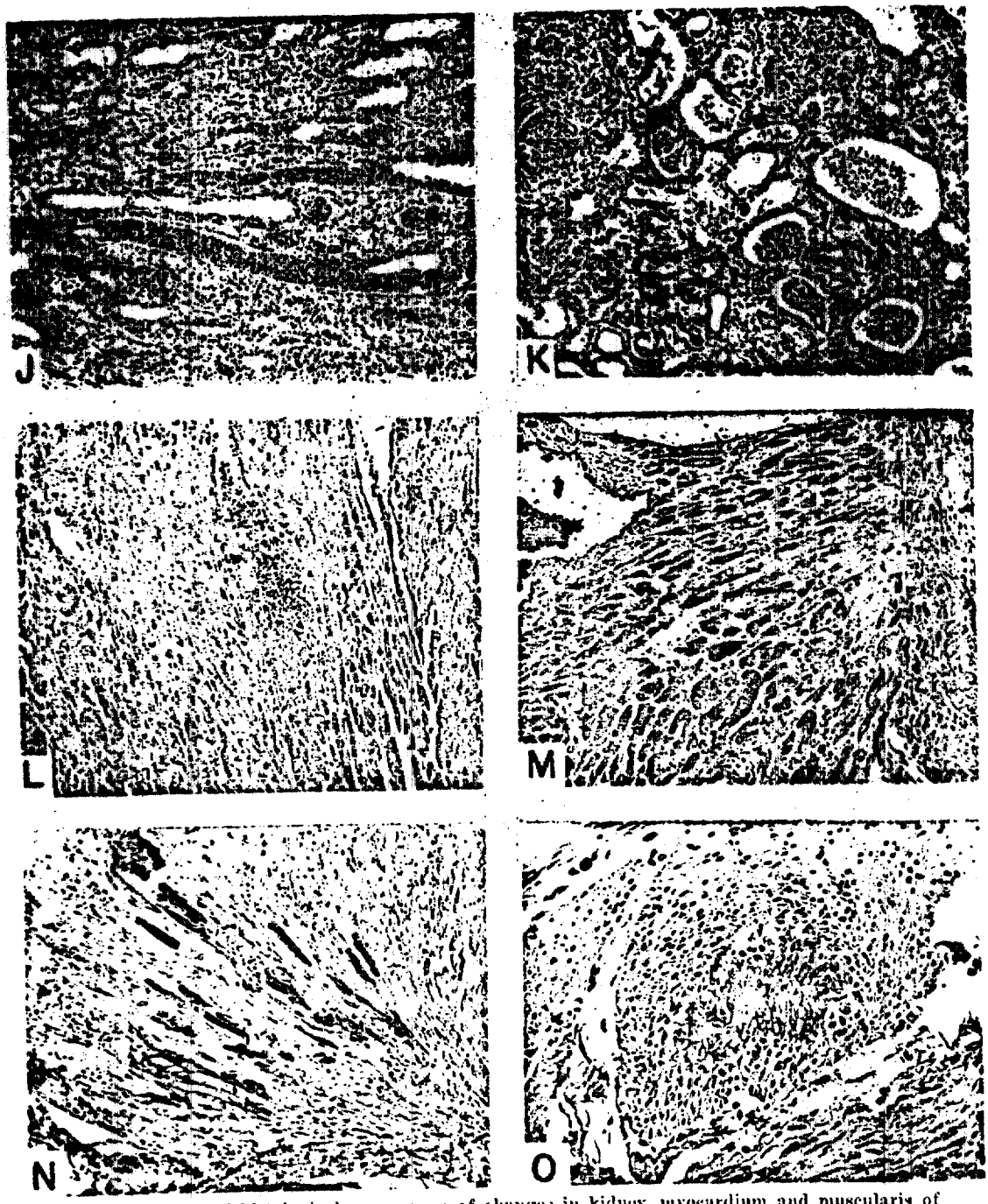

FIG. 4. Typical histological appearance of changes in kidney, myocardium and muscularis of stomach in alpha-tocopherol fed albino rats following renal injury. (Animals 1-13 of Table I.)

J. Ascending pyelonephritis in renal medulla. Note large tubular casts composed of polymorphonuclear leucocytes and interstitial infiltration.

K. Typical lesion of renal cortex. Showing tubular dilatation with casts of pus cells, interstitial inflammation, necrosis and calcification of tubular epithelium and foci of tubular regeneration.

L. Interstitial myocarditis. Diffuse infiltration and focal collection of predominantly mononuclear cells.

M. Focus of myocardial necrosis. Note darkly stained necrotic fibers in left lower corner of photograph.

N. Myocardium. Necrosis and calcification of individual muscle fibers.

O. Stomach wall. Showing necrosis and calcification in muscularis.

guish specific substitution effects in deficiency from pharmacodynamic effects obtained with relatively large dosages in the nondeficient organism. Most clinical applications of vit. E obviously belong to the latter group. In this connection it may be of interest to quote the chief proponents of vit. E therapy in cardiovascular disease(24): "Vit. E therapy in our hands is not substitution therapy but a form of chemotherapy. This is a point that deserves emphasis. The doses we use are larger than nutritional studies would demand, but usually smaller dosage is ineffectual. We think of alpha-tocopherol as a chemical compound which also happens to be a food constituent, but whose dosage level in established cardiac disease is not closely related to that coincidence. . . . A small dosage of E gives but little hint of what it can accomplish in massive dosage." We are in agreement and believe that our experimental studies have tested the drug effect of alpha-tocopherol in massive dosage, rather than a specific vitamin action.

In search for an explanation of the detrimental drug effect of alpha-tocopherol we were intrigued by the view of Lecoq *et al.*(25), who ascribe alkalizing properties to vit. E. They demonstrated that a permanent increase in nerve chronaxia due to hypervitaminosis E could be brought back to normal by acidification with the help of ammonium chloride. Since it was demonstrated by one of us(26) that ammonium chloride is capable of inhibiting and even preventing emergence of cardiovascular necrosis in the albino rat, we are now investigating the possibility that an alkalizing action of the vitamin might have been responsible for its deleterious influence in our experiments.

Summary. 1. Disseminated muscular necrosis following experimental nephropathy in albino rats maintained on complete rations, was not beneficially influenced by the presence of excessive amounts of alpha-tocopherol. On the contrary, this drug appeared responsible for a higher incidence of advanced lesions in the aorta, coronary arteries, and stomach. 2. Concomitant renal hypertension was not alleviated by the presence of alpha-tocopherol in excess. In normotensive rats large dosages of this compound may induce moderate hypertension.

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EXPERIMENTAL SENSITIZATION TO VITAMIN E (TOCOPHEROL) IN GUINEA PIGS[1]

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Experimental attempts have been made to induce vitamin deficiencies by immunization methods. Some vitamins act as coenzymes in metabolic pathways, and it was thought possible to interfere with these pathways by the production of specific neutralizing antibodies to the vitamin.

Since vitamin E deficiency is believed to have some association with muscular dystrophy, attempts have been made to produce muscular changes in guinea pigs associated with vitamin E deficiency. It was subsequently learned that guinea pigs may not be the animal of choice for the effect sought.

However, a delayed sensitization to vitamin E was encountered which occurred not only following the incorporation of the vitamin in Freund's adjuvants, but also by intracutaneous injection with undiluted vitamin alone. In the latter instance the vitamin E acted as the sensitizing as well as the skin test material.

Vitamin E, or tocopherol, is of interest in sensitization studies because it can be obtained both by extraction from natural sources or by synthesis in the laboratory. Thus, problems regarding sensitization to inpurities rather than to the vitamin can be controlled.

Also of interest is the chemical structure of vitamin E, which is in a form that can shift to become a quinoid structure. The resulting quinone is chemically active and may react with the body protein, thus becoming haptenic. This reactivity of certain chemicals has been postulated as the mechanism by which such compounds become sensitizing (1).

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[2] Career Development Award of the National Institute of Allergy and Infectious Diseases, Grant 5-K3-AI-14920.

MATERIALS AND METHODS

Hartley strain guinea pigs of either sex and ranging in size from 400 to 850 g were obtained from either Tumblebrook Farms, Camm Research Institute, or from Harlan Small Animal Industry, and fed Purina guinea pig chow and given water *ad libitum*.

Three different materials were used for sensitization or skin test. These were: the natural *d*,α-tocopherol, the synthetic *dl* isomer and the synthetic *dl*,α-tocopheryl acetate. All of the compounds were considered pure.[3]

Two methods were used for sensitization: *a*) The vitamin E was incorporated in complete Freund's adjuvant with killed mycobacteria. The method of emulsion preparation has been previously described (2). Each guinea pig received intradermally in multiple sites 0.4 ml of emulsion containing a total of 20 mg of vitamin E and 0.8 mg of killed mycobacteria. Controls were injected with a comparable emulsion without the vitamin E. *b*). Guinea pigs were injected intradermally with 10 to 20 mg of tocopherol or the acetate derivative. Subsequent skin tests were carried out by injecting 0.01 to 0.02 ml of the undiluted tocopherol intracutaneously. Uninjected animals were used as skin test controls. Where possible the skin tests were done on the animal's side contralateral to the first injection. Skin test sites were examined and measured 2 to 4 hr after injection and again at 24, 48 and 72 hr and 4 days after injection. The degree and extent of erythema, discoloration, swelling (whether soft or hard), induration, presence of necrosis and scab were recorded. In the tables, however, the reactions are graded as follows: ++++, over 15 mm dark pink and indurated; +++, 12 to 15 mm dark pink and indurated; ++, 9 to 11 mm pink and indurated;

[3] Obtained through the courtesy of Dr. Charles D. Robeson, Distillation Products Industry.

TABLE I

Sensitization to vitamin E (d,α-tocopherol) by emulsification of vitamin E in complete Freund's adjuvant

Sensitizing Material Injected	Skin Test Readings at 26 Days with d,α tocopherol		
	24 Hr	48 Hr	72 Hr
Vitamin E (d,α-tocopherol) complete Freund's adjuvant	+	+++	++++
	++	+++	+++
	+	++	+++
	0	+	+
Complete Freund's adjuvant	0	+	++
	0	+	+
	0	+	+
	0	0	0

+, 7 to 8 mm pink and indurated; 0, 0 to 6 mm faint pink or colorless nodule.

RESULTS

In Table I are presented the data on sensitization with 20 mg of d,α-tocopherol in complete Freund's adjuvant. It can be seen from these data that at 26 days on skin testing with d,α-tocopherol, four out of four animals reacted to the skin test material with moderate reaction. In a control group consisting of animals injected with complete adjuvant without tocopherol there was some reaction which will be discussed shortly. Uninjected control guinea pigs were negative when examined at 24, 48 and 72 hr after skin testing.

It can be seen from Table II that in retesting the animals in Table I at 71 days with the dl,α-tocophorol, those injected with d,α-tocopherol and adjuvant showed moderate reaction to the dl,α-tocopherol but only questionable reactions to the dl,α-tocopheryl acetate. In the group which received complete adjuvant without tocopherol but were skin tested at 26 days (15 days previous to the 71st-day skin test) the dl,α-tocopherol and dl,α-tocopheryl acetate responses were negative. The three uninjected control animals skin tested with d,α-tocopherol were negative. Six guinea pigs skin tested with the dl compound were negative and one showed a 2+ reaction. All five of the dl,α-tocopheryl acetate controls were negative. These data are evidence of the ability to sensitize to d,α-tocopherol by the injection of tocopherol emulsified in complete Freund's adjuvant.

It is of interest that some of the animals injected with complete Freund's adjuvant emulsion alone showed mild positive reactions to tocoph-

TABLE II

Sensitization to vitamin E (d,α-tocopherol) by use of complete Freund's adjuvant

	Skin Testing with dl, α-Tocopherol and dl, α-Tocopheryl Acetate			
Sensitizing material	Day of first skin test	Day of retest	Skin test material*	Reactions 48–72 Hr.
d,α-Tocopherol complete Freund's adjuvant	26	71	d-Alpha	+++, +++, ++, ++
			dl-Alpha	++, ++, ++, +
			dl-Acetate	±, ±, ±, 0
Complete Freund's adjuvant	26	71	d-Alpha	++, ++, +, 0
			dl-Alpha	0, 0, 0, 0, 0
			dl-Acetate	0, 0, 0, 0
Uninjected controls	26		d-Alpha	0, 0, 0
Uninjected controls	71		dl-Alpha	++, 0, 0, 0, 0, 0, 0
			dl-Acetate	0, 0, 0, 0, 0

* d-Alpha = d,α-tocopherol; dl-Alpha = dl,α-tocopherol; dl-Acetate = dl,α-tocopheryl acetate

TABLE III

Sensitization to vitamin E following the injection of complete Freund's adjuvant without vitamin E. Guinea pigs skin tested with 0.02 ml d,α-tocopherol 21 days following initial injection

Group Injected With	Day 1	Day 2	Day 3	Day 4	Day 7	Day 8	Day 9	Day 10	Day 11	Day 14
0.7 ml complete Freund's adjuvant	0	+	+	±	0	0	0	0	0	0
	0	++	++	++	+++	+++	+++	+++	+++	0
	0	++	++	++	++++	++++	++++	++++	++++	+++
0.7 ml incomplete Freund's adjuvant	0	0	0	0	+	+	+	0	±	0
	0	0	0	0	+++	++++	+++	+++	+++	+
	0	0	0	±	±	±	+	+	0	0
Uninjected	0	0	0	0	0	+	+	+	+	+
	0	0	0	0	++	+++	+++	+++	+++	++
	0	0	0	0	0	0	+	0	0	0

TABLE IV

The induction of sensitization to tocopherols, d,α-tocopherol, dl,α-tocopherol and dl,α-tocopheryl acetate by intracutaneous injection. Genesis of skin test lesions at original sensitizing site

Material Injected	Day 1	Day 2	Day 3	Day 5	Day 6	Day 7	Day 8	Day 10
d,α-Tocopherol	0	0	+	++	++	++++	++++	+++
	0	0	+	±	++	+++	+++	+++
	0	0	+	+	++	++	+++	++++
	0	0	+	±	+++	+++	+++	+++
	±	+++	++++	+++	+++	++	0	
dl,α-Tocopherol	0	0	0	+	++	+	+	
	0	0	++	+	+++	+++	++	
	0	0	+	+	++	++	+++	0
	0	0	±	±	+	++	+++	++++
	0	0	++	+	++	+	++	++
dl,α-Tocopheryl acetate	0	0	0	0	0	+	+	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	+	+	+	+
	0	0	0	0	0	0	0	0

tol. This may be due to the presence in killed tycobacteria of a compound very similar in tructure to d,α-tocopherol. Vitamin K-like mpounds have been demonstrated in tubercle acilli by Noll (3). Another experiment (Table III) testing the efficacy of the Freund's adjuvant thout tocopherol for sensitization to tocopherol gave essentially the same results as in Table 1. Reactions to d,α-tocopherol are presented in Table III, which includes the 24-hr and 14-day skin test readings. It can be seen that the tubercle bacilli in the complete adjuvant are responsible for the sensitization to tocopherol since groups injected with incomplete adjuvant and the uninjected controls are essentially negative 24 to 48 hr following skin testing.

It was observed on skin testing of normal animals that, following the intradermal injection of tocopherol, a colorless or faint pink nodule appeared which persisted for about 4 days. After 4 days the reaction increased in size with changed character in regard to erythema and induration.

TABLE V

Sensitization to tocopherol; d,α-tocopherol, dl,α-tocopherol, dl,α-tocopheryl acetate by the intracutaneous method

Sensitising Material	Skin Testing Compound*	Reaction 48 Hr (Test on Day 8)
d,α-Tocopherol	*d*-Alpha	++, ++
	dl-Alpha	++, ++
	dl-Acetate	±, ±
dl,α-Tocopherol	*dl*-Alpha	+++, +++, ++
	d-Alpha	+++, 0
	dl-Acetate	±, ±, 0
dl,α-Tocopheryl acetate	*dl*-Acetate	±, ±, ±, 0, 0
	d-Alpha	++, +, +, +, 0
	dl-Alpha	++, ++, ++, ++, 0
Normal controls	*d*-Alpha	++, 0, 0, 0, 0, 0, 0, 0
	dl-Alpha	++, 0, 0, 0, 0, 0, 0
	dl-Acetate	0, 0, 0, 0, 0

* For explanation of testing compounds see footnote to Table II.

By the 8th day a well-defined pink area was observed, with induration and perhaps a necrotic center.

In Table IV are presented the results following the intracutaneous injection of *d*,α-tocopherol, *dl*,α-tocopherol and *dl*,α-tocopheryl acetate. 0.01 to 0.02 ml was used for the skin test. The genesis of the lesions are shown for from 1 to 10 days following primary injection. It can be seen that *dl*,α-tocopheryl acetate is not very reactive, whereas *d*,α-tocopherol and *dl*,α-tocopherol were active in inducing reactions in the initial site of skin testing.

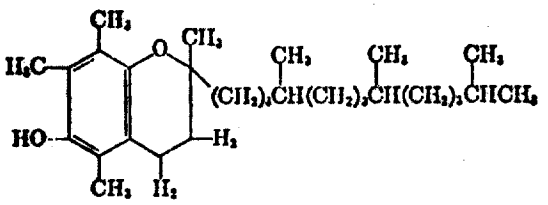
Animals in this experiment were skin tested in multiple sites at Day 8 with various test materials including *d*,α-tocopherol, *dl*,α-tocopherol and *dl*,α-tocopheryl acetate. In this instance the skin reactions to the second injection reached their peak at about 48 hr and are given in Table V in abbreviated form. It can be seen that *dl*,α-tocopheryl acetate sensitized to both *d*,α-tocopherol and *dl*,α-tocopherol, but with questionable sensitization to *dl*,α-tocopheryl acetate, whereas *dl*,α or *d*,α-tocopherol sensitized to *d*,α and *dl*,α with questionable sensitization to *dl*,α-tocopheryl acetate. In the normal controls one animal out of eight and one out of seven showed reactions to the primary injection of *d*,α and *dl*,α-tocopherol, respectively.

DISCUSSION

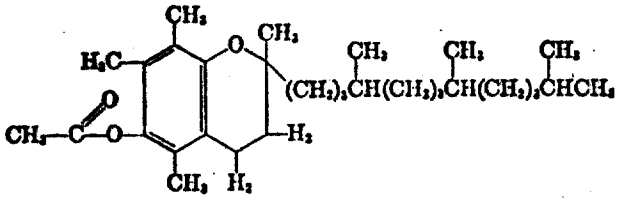
Vitamin E, or α-tocopherol, is a light oily yellow liquid, fat-soluble and water-insoluble, but dispersible in aqueous medium in the presence of a surface active agent. We have used two isomers of tocopherol: the dextrorotatory form which is the natural isomer (optically active) obtained by extraction and distillation, and the synthetic *dl*,α-tocopherol (optically inactive). *dl*,α-Tocopheryl acetate compound is also a synthetic material. With these materials it is possible to sensitize with one form and skin test with the other, thereby ruling out reaction to impurities in the preparations.

Sensitization with vitamin E incorporated in Freund's adjuvant is not unexpected. The sensitization following the intracutaneous injection of pure vitamin E alone was unexpected. It can perhaps be explained as follows: the role of the quinone structure in inducing sensitization to simple chemical compounds has been postulated (2). It has been shown that the quinone structures can attach to proteins or bind to proteins. In the situation which we have presented, the tocopherol although not initially in quinone structure can become quinone when injected into the skin. Oxidation *in vivo* can account for the binding of the vitamin E with body protein. The lipids of the skin may also play a role. The

α-Tocopherol



α-Tocopheryl acetate



α-Tocoquinone

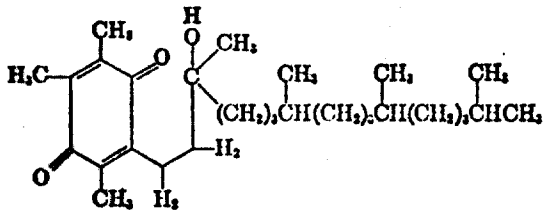


Figure 1

oily nature of the vitamin E and its insolubility may account for its persistence in the tissues for a period of time which permits it to play the dual role of a sensitizing and a skin test material. This would be in keeping with an observation of Chase (4) that on the 7th or 8th day following intracutaneous injection of some chemical compounds, a flare reaction may be seen at the site, due to the reaction with remaining traces of the sensitizing material.

Figure 1 shows the structure of α-tocopherol and its quinone derivative.

Also included is a structural diagram of α-tocopheryl acetate in which the hydroxyl group attached to carbon 6 is replaced by an acetate group. The structure of tocopheryl acetate becomes more stable so that it is more difficult to make a quininoid structure. This is in keeping with the results reported in Table V, which show that injection of *dl*,α-tocopheryl acetate does not yield a primary sensitization as good as that obtained by *d*,α-tocopherol or *dl*,α-tocopherol.

It is of interest to note that the *dl*,α-tocopherol produces a higher degree of sensitization, especially when tested with *dl*,α-tocopherol, than does the *d*,α-tocopherol (the natural form).

To be explained is the lack of skin test reactivity with *dl*,α-tocopheryl acetate following sensitization with the *d*,α-tocopherol and the *dl*,α-tocopherol. On the other hand, *dl*,α-tocopheryl acetate, when used as a primary injection, yields animals which are sensitive to *d*,α-tocopherol and *dl*,α-tocopherol, although mild or negative reactions appear in the *dl*,α-tocopheryl acetate group. A possible explanation for this is that the skin reaction which is observed following *d*,α-tocopherol sensitization is to the postulated "hapten" protein combination and not to the "hapten" itself.

Chase and Battisto (5) have demonstrated that by feeding certain simple chemical compounds it is possible to interfere with experimental sensitization to those compounds. It might be expected that since our guinea pigs receive tocopherol daily in their diet they might become desensitized. This is not the case.

SUMMARY

The delayed or tuberculin type of sensitization has been produced in guinea pigs (as shown by skin test) using the natural compound vitamin

E (*d*,α-tocopherol) incorporated into complete Freund's adjuvant.

D,α-tocopherol or *dl*,α-tocopherol have been shown to be sensitizing when injected into the skin in quantities of 0.01 to 0.02 ml (approximately 10 to 20 mg). The skin site acts both as a sensitizing site and a skin test site on primary inoculation.

A vitamin E-like compound has been demonstrated immunologically in tubercle bacilli. This is indirectly observed by skin testing with pure α-tocopherol animals which have been injected with Freund's complete adjuvent less the tocopherol.

Although the guinea pig is constantly coming in contact by the alimentary route with tocopherol in its food, this does not desensitize the animal.

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THE INFLUENCE OF VITAMIN E ON BARBITURATE NARCOSIS

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Padua Section -- Session of August 8, 1957

In the course of some investigations on the influence of vitamin E on the alterations of the hepatic cell caused by barbiturate narcosis, I had occasion to observe that, in animals treated precedingly with α-tocopherol, the dose of narcotic necessary to achieve the narcosis was smaller than that necessary in untreated animals (1).

Given the consistency of this report, it seemed to me to be of a certain experimental interest to study the possible relations that exist between this vitamin and the development of barbiturate narcosis.

Toward such an end, experiments were undertaken on 12 dogs of approximately equal size, 6 of which (controls) were anesthetized only; the other 6, on the contrary, were submitted before the narcosis to a treatment with vitamin E, injected parenterally in a dose of 100 mg for three consecutive days. A fourth dose of vitamin, but of 100 mg, was injected 2 hours before the anesthesia.

Two different barbiturates were used: pentothol and farmotol. 20 ml of solution at 2.5%, equal to .5 g of one of the two barbiturates, were administered to each animal. This solution was injected slowly via the intravenous route until a surgical narcosis was reached, and then in succeeding doses as necessary until exhaustion, taking care to keep the animal in a deep narcosis without, however, arriving at a state of apnoea.

The absence of corneal reflexes and the absence of muscular reactions of defense against painful stimuli served as criteria of the narcosis.

The partial doses administered, the behavior of the animal, and the duration of the anesthesia were strictly recorded.

Results. -- Part of the results are reported in the following table. (see next page)

From the records of the examinations it is revealed that:

1) in the animals treated with vitamin E, the doses of barbiturate necessary to achieve a deep narcosis are lower than those needed for the corresponding untreated control animals;

2) in the animals treated with vitamin E, in order to maintain the state of narcosis afterward, succeeding administrations of barbiturate in doses larger than those given to the

controls are necessary;

3) the same animals, but injected with larger doses of anesthetic, tend toward a more rapid revival;

1. 2.

Cane N.	Animali non trattati con vit. E a) Dose di sol. 2,5% necessaria per raggiungere la narcosi, ml	b) Durata totale della narcosi con ml 20 di sol. al 2,5%, min	Cane N.	Animali trattati con vit. E a) Dose di sol. 2,5% necessaria per raggiungere la narcosi, ml	b) Durata totale della narcosi con ml 20 di sol. al 2,5%, min
1	10,5	55	1	8,0	55
2	11,0	63	2	7,5	53
3	10,0	60	3	8,5	60
4	10,5	60	4	7,5	55
5	10,5	65	5	8,0	58
6	9,5	70	6	7,5	60

Key:
1.= animals not treated with vitamin E.
2.= animals treated with vitamin E.
a)=dose of 2.5% solution necessary to reach narcosis (ml)
b)=totalduration of narcosis after 20 ml of 2.5% solution (in minutes)

4) the total duration of the narcosis in the case of the administration of an equal quantity of narcotic (0.5 in 20 ml) is slightly lower in the vitaminized animals than in the controls.

No difference was noted in the case of the animals narcotized with pentothol as compared to those treated with farmotol.

In no animal was the progress of the narcosis disturbed by secondary actions. The animals treated with vitamin and those not treated revealed in this regard the same behavior.

Considering the sum of these experiments on the influence of vitamin E on barbiturate narcosis, we can observe how the vitamin renders the animal initially more sensible to the action of the anesthetic and at the same time offers the possibility of a more rapid revival, as well as the fact that it was necessary to administer the barbiturate more frequently than to the control animals in order to maintain the state of narcosis.

It is difficult at this time to be able to establish the nature of the mechanism with which vitamin E is able to influence the development of barbiturate anesthesia, be it because of the complexity of the mechanism of action of the barbiturates on the nervous system, which is not yet clear in every way, or be it because of the multiplicity of actions attributed to vitamin E, some of which are still the subject of divergent opinions.

Nonetheless, since a hepatoprotective action is recognized in vitamin E, the supposition, that it may interfere as well in the metabolism of barbiturates, accelerating their catabolization, should not be excluded.

(1) Acta Anaesthesiologica, in print

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THE ABSORPTION OF α-TOCOPHEROL IN CONTROL SUBJECTS AND IN PATIENTS WITH INTESTINAL MALABSORPTION

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SUMMARY

1. The absorption of physiological doses of tritiated α-tocopherol has been studied in seven control subjects and in twenty-three patients with malabsorption due to various causes.

2. Control subjects absorbed between 55·0 and 78·6% of an oral dose. Radioactivity appeared in the plasma between 1 and 3 hr, reached a peak between 5 and 9 hr, and subsequently fell exponentially with a mean half-life of 53 hr. Radioactivity in plasma was almost all associated with free α-tocopherol.

3. Absorption of α-tocopherol was most severely impaired in patients with biliary obstruction, and somewhat less severely impaired in patients with exocrine pancreatic insufficiency. Malabsorption in patients with an abnormal small intestinal mucosa varied with the severity of the condition, and could be correlated with the degree of steatorrhoea.

4. In two patients with intestinal lymphangiectasia, malabsorption of the vitamin was relatively more severe than that of fat. Mild malabsorption was found in five of the six patients who had had a partial gastrectomy.

5. The amount of radioactivity recovered from the urine in 3 days ranged from 0·0 to 21·4% of the dose given and could be correlated with the degree of absorption.

Deficiency of vitamin E in man is well recognized, and although it does not appear to cause any clearly defined clinical syndrome, certain biochemical, haematological and histological abnormalities have been demonstrated in subjects who are depleted of the vitamin either because of poor dietary intake (Horwitt, 1960) or because of malabsorption (Binder *et al.*, 1965). These abnormalities include an enhanced susceptibility of the red cell to an oxidant stress *in vitro* (Rose & György, 1952), an increase in urinary creatine excretion (Nitowsky *et al.*, 1962) and the deposition of ceroid pigment in smooth muscle (Pappenheimer & Victor, 1949).

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A deficiency of vitamin E has been described in premature infants (Oski & Barness, 1967) and occasionally in subjects suffering from dietary malnutrition (Rahman *et al.*, 1964). It seems to occur most frequently, however, in patients with long-standing steatorrhoea due to many causes, including coeliac disease (Darby, Cherrington & Ruffin, 1946), fibrocystic disease and biliary atresia (Nitowsky, Gordon & Tildon, 1956), chronic pancreatitis, Whipple's disease, intestinal resection, jejunal diverticulosis and hepatic cholestasis (Binder *et al.*, 1965) and after gastric resection (Leonard, Losowsky & Pulvertaft, 1966).

Despite the frequent demonstration of vitamin E deficiency in patients with steatorrhoea, there have been few reports on the characteristics of its absorption either by normal subjects or by patients with malabsorption. Klatskin & Molander (1952) measured faecal excretion of vitamin E by normal subjects and by patients with cirrhosis and calculated net absorption from an estimated dietary intake. They also measured the net absorption of pharmacological doses of vitamin E given orally, and followed the rise in plasma concentration of the vitamin at time intervals after its ingestion. Similar techniques have been used by several other workers (Darby *et al.*, 1946; Week, Sevigne & Ellis, 1952; Pomeranze & Lucarello, 1953; Pelkonen, 1963; Schmandke & Schmidt, 1968); but it is not possible to measure the absorption of physiological doses of the vitamin by such methods. More recently Blomstrand & Forsgren (1968) have studied the absorption of isotopically-labelled vitamin E into the lymph of two patients with malignant disease who had had cannulae inserted into their thoracic ducts. In these studies no attempt was made to measure total absorption.

In this paper we report the characteristics of the absorption of physiological amounts of tritiated α-tocopherol by patients with and without intestinal malabsorption.

SUBJECTS AND METHODS

Subjects studied

The absorption of vitamin E was measured in seven control subjects, who were convalescent in hospital and who had no evidence of malabsorption, and in twenty-three patients with malabsorption (Table 1). Six of the patients with malabsorption had adult coeliac disease, one had tropical sprue, and two had lymphangiectasia, the diagnosis being made in all cases by jejunal biopsy. Three patients had chronic pancreatic insufficiency diagnosed by pancreatic function tests, and five had obstruction of the common bile duct, proven by laparotomy. None of the subjects was given vitamin E before the absorption test was performed. The nature of the investigation was explained in detail to the patients and all agreed to take part in the study.

Methods

Materials used and their purity. Unesterified tritiated DL α-tocopherol, specifically labelled in the 5 methyl group (specific activity 1725 mCi/mM) was obtained from the Radiochemical Centre, Amersham, and stored *in vacuo* or under nitrogen at −20°. The tocopherol content of the material supplied was assessed at the Radiochemical Centre by U.V. absorption and it was checked in our laboratory, by the method of Martinek (1964). Five months after the batch had been prepared, the tocopherol content had fallen to 88% of that specified at the time of labelling.

The radiochemical purity was checked by thin-layer chromatography approximately once a month. 0·2 μCi of α-tocopherol, dissolved in 0·125 mg of unlabelled tocopherol and protected against oxidation by the addition of 0·25 mg of butylated hydroxytoluene (BHT), was applied

thin layer of silica gel G. The plate was developed either in benzene or in 10% acetone in benzene. The silica gel was then divided into 2 cm strips, and scraped directly into scintillation vials. The percentage of the applied radioactivity recovered from the zone corresponding to a marker of α-tocopherol was 98% initially and declined 1–2% per month. The isotope was not used after the radiochemical purity had fallen below 87%.

Procedure of absorption test. Fasting subjects were given between 12 and 25 μCi of tritiated α-tocopherol in 1 mg of unlabelled vitamin dissolved in propylene glycol. The dose was delivered directly on to the tongue from a weighed syringe and rinsed down with 150 ml of milk. Heparinized blood samples were taken at hourly intervals between 0 and 6 hr, and at 9, 12, 24, 72 and 120 hr after the dose had been given. Plasma was separated and stored at −15°. Faeces were collected for 6 days and urine for 3 days. Faeces were homogenized and aliquots of these and of the urine collections were stored at −15°. Specimens were assayed for radioactivity within 1 week of collection.

Measurement of plasma radioactivity. 2 ml of plasma were shaken with 40 ml of chloroform–methanol (2:1) and the phases were separated with 8 ml of water. The chloroform layer was dried down under a stream of nitrogen and the residue redissolved in 10 ml of toluene, containing 0·4% PPO (2.5-diphenyl-oxazole) and 0·01% POPOP (1,4-bis (2-(5-phenyloxazolyl)) benzene) and transferred quantitatively into counting vials. Radioactivity was measured using a Packard Tricarb Scintillation counter. In later experiments the radioactivity was counted in butyl PBD, 6 g/l of toluene ((2-4 butylphenyl)-5(4 biphenylyl)-1,3,4,-oxidiazole) and counted in a Beckman Scintillation counter. The efficiencies of the two methods of counting were 40% and 57·5% respectively. Quenching of radioactivity by plasma was negligible except when it contained excess bilirubin. Quenching was corrected either by adding a small amount of known radioactivity as an internal standard or by using an automatic external standard. Results were expressed as

$$\frac{\text{Disintegrations min}^{-1}\text{ ml of plasma}^{-1}}{\text{Dose in }\mu\text{Ci kg body weight}^{-1}}$$

Peak counts in plasma from control subjects varied between 1793 and 490 cpm, and counts at 72 hr between 480 and 840 cpm. Background counts varied between 20 and 40 cpm.

Two experiments in which small quantities of labelled α-tocopherol were added to plasma, incubated for 30 min at 37°, and then extracted with chloroform–methanol showed a recovery of 94·5% and 98·4% of the isotope.

Nature of plasma radioactivity. The radioactivity in lipid extracts of plasma was analysed by thin-layer chromatography on silica gel G. Two ml of plasma were shaken with 40 ml of chloroform–methanol (2:1) and the phases separated with 8 ml of water. The chloroform layer was dried down and the residue redissolved in hexane and an aliquot applied to a thin layer plate of silica gel G, with 0·125 mg of unlabelled α-tocopherol acting as a carrier, and 0·25 mg of BHT acting as an anti-oxidant. After development with 10% acetone in hexane the silica gel was divided into 2 cm strips which were scraped into scintillation fluid and counted, with an external standard to correct for quenching.

Faecal radioactivity. Each collection of faeces was homogenized with water and an aliquot of approximately 5 g was refluxed for two periods of 2 hr with 200 ml of acetone–methyl alcohol (1:1). The extract was dried in a rotary evaporator and the residue dissolved in 5 ml of ethanol. Aliquots of 0·1 ml and 0·2 ml were transferred to counting vials and dissolved either in toluene

containing POPOP or in butyl PBD. Radioactivity was assessed by the method used for the plasma samples. Three recovery experiments were performed in which known amounts of the isotope were added to the faeces. The mean recovery was 96·7% (range 92·4–101·3%).

Nature of faecal radioactivity. An aliquot of approximately 5 g of faeces was extracted with chloroform–methanol (2:1). The amount of water-soluble radioactivity was assessed by drying down 10 ml of the methanol layer in a rotary evaporator and redissolving the residue in butyl PBD scintillation fluid (butyl PBD 5 g, naphthalene 80 g, ethoxyethanol 400 ml and toluene 600 ml) (counting efficiency 44%). The chloroform layer was dried down, redissolved in hexane and the distribution of radioactivity was assessed by thin-layer chromatography using the method described previously for plasma.

Urinary radioactivity. In early experiments 0·4 ml of each day's urine was counted in 10 ml of toluene POPOP and 3 ml of ethyl alcohol using a Packard Tricarb counter (counting efficiency 21%) and in later experiments in 10 ml of butyl PBD using a Beckman counter.

Plasma tocopherol. Tocopherol concentrations in samples of plasma from fasting patients were measured by the colorimetric method of Martinek (1964).

Faecal fat. Faecal fat was measured by the method of Van de Kamer, ten Bokkel Huinink & Weyers (1949).

RESULTS

Control subjects

Seven control subjects were studied (Table 1). None had steatorrhoea and all had normal levels of plasma tocopherol, ranging from 614 to 1151 μg/100 ml (normal range in our laboratory 400–1250 μg/100 ml).

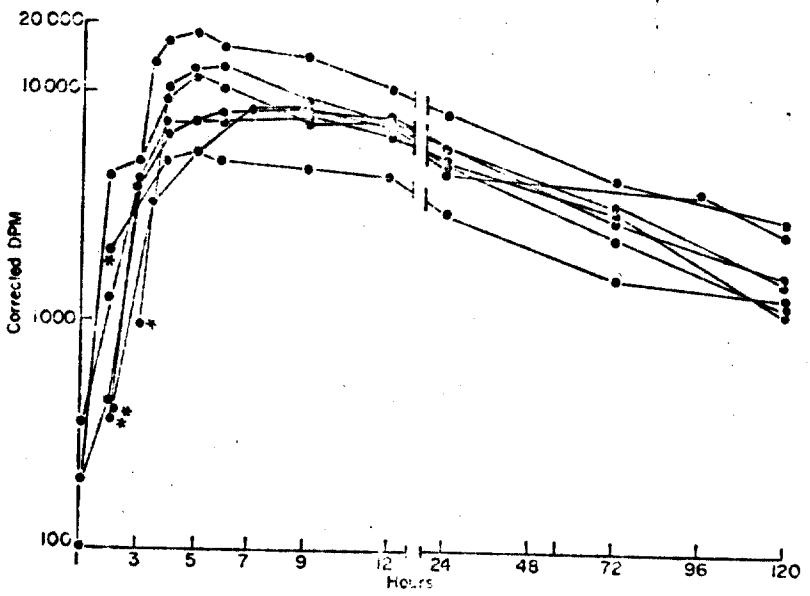

FIG. 1. Plasma radioactivity following an oral dose of [^{2}H]DL-α-tocopherol in seven control subjects. DPM/ml plasma corrected for dose and body weight. * Less than 100 DPM/ml plasma up to this time.

Plasma radioactivity. Plasma radioactivity in the control subjects at varying time intervals after receiving the standard dose of tritiated vitamin E is shown in Fig. 1. Radioactivity first appeared in the plasma between 1 and 3 hr after the oral dose. Radioactivity was maximal between 3 and 9 hr, and thereafter it declined exponentially, with a mean plasma half life of 53 hr (range 42–69 hr).

Net absorption. The absorption of tritiated α-tocopherol was calculated by assuming that the administered radioactivity not recovered from the faeces excreted in 6 days had been absorbed. A mean of 68·6% of the oral dose was absorbed by control subjects (range 55·0–78·6%) (Fig. 2).

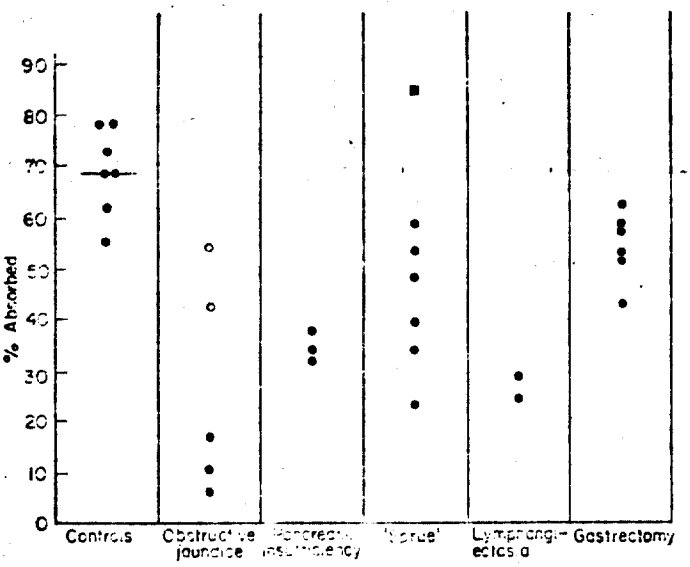

FIG. 2. Net absorption of [^{3}H]DL-α-tocopherol by control subjects and patients with malabsorption. 'Sprue' patients include six with adult coeliac disease and one with tropical sprue. ■, One patient with adult coeliac disease who had had intermittent treatment with a gluten-free diet. ○, Faecal collections for only 3 days.

Urinary radioactivity. A mean of 8·2% (range 3·4–16·1%) of the radioactivity administered orally was recovered from the urine in the next 3 days (Table 1). Of the total radioactivity recovered from the urine, a mean of 36·6% was found in the first day, 38·6% in the second day and 24·8% in the third day.

Patients with malabsorption

Malabsorption due to impaired intraluminal digestion. The net absorption of tritiated vitamin E was severely reduced in five patients with obstructive jaundice of whom four had carcinoma of the head of pancreas and one had carcinoma of the bile ducts (Fig. 2). Three of the patients absorbed only 6·0%, 11·0% and 16·7% of the dose. Faeces were collected for only 3 days from the other two patients. In this time they excreted 46·3% and 58·1% of the administered dose compared with a maximum excretion of 45·0% over 6 days by a control patient. No radioactivity was found in the plasma of the two patients with the most severe malabsorption (J.H.

TABLE 1. Faecal fat excretion, plasma tocopherol concentration, net absorption of [^{3}H]α-tocopherol and urinary excretion of radioactivity in the patients studied

Patient	Age	Sex	Diagnosis	Faecal fat (g/day)	Plasma tocopherol (μg/100 ml)	Net absorption tocopherol (% dose)		Urinary excretion radioactivity (% dose)	
I. Control patients with normal fat absorption									
E.G.	65	F	Gastric ulcer	2·2	1151	55·0	Mean 68·6%	3·4	Mean 8·2%
V.R.	49	F	'Spastic colon'	3·9	727	78·6		7·9	
R.P.	42	M	Duodenal ulcer	3·1	1000	67·1		3·4	
J.S.	41	M	Raynaud's phenomenon	6·0	614	72·5		5·5	
P.H.	48	M	Hypertension	3·0	918	67·3		11·8	
H.J.	56	M	Dietary scurvy	5·4	668	61·3		16·1	
H.V.	58	M	Gastric ulcer	6·0	857	78·5		9·4	
II. Pancreatic and biliary disease									
J.B.	58	M	Ca bile duct	60·0	Not determined	11·0	Mean 11·2*	0·0	Mean 0·5%
D.T.	53	F	Ca head of pancreas	16·9	Not determined	53·7*		1·4	
E.Gw.	54	F	Ca head of pancreas	14·1	468	41·9*		0·8	
I.Gar.	57	M	Ca head of pancreas	8·1	550	16·7		0·4	
J.H.	59	M	(a) Ca head of pancreas	39·5	915	6·0		0·0	
			(b) After by-pass	5·5	Not determined	48·7		4·3	
C.P.	71	M	Chronic pancreatitis	25·0	126	33·9	Mean 34·7%	2·7	Mean 4·5%
F.K.	68	F	Pancreatectomy and gastro-jejunonostomy	16·3	170	31·9		7·1	
E.H.	51	M	(a) Chronic pancreatitis and partial gastrectomy	35·5	Not determined	38·2		3·7	
			(b) On cotazym	Not determined	156	59·5		7·3	
III. Jejunal mucosal abnormalities									
F.M.†	39	M	Adult coeliac disease	7·1	747	84·6		21·4	
C.C.	50	M	Adult coeliac disease	44·5‡	645	23·1	Mean 42·6%	0·0	Mean 4·5%
E.S.	72	F	Adult coeliac disease	11·0	414	52·7		5·6	
W.J.	46	M	Tropical sprue	40·0	334	34·1		1·8	
H.W.	59	M	Adult coeliac disease	8·0	203	58·5		5·3	
J.B.	30	M	Adult coeliac disease	20·1	137	39·2		11·7	
E.A.	58	F	Adult coeliac disease	24·2	107	47·8		2·4	
IV. Lymphatic abnormalities									
B.M.	20	M	Intestinal lymphangiectasia	13·8	187	28·5	Mean 29·5%	2·3	Mean 1·8%
J.W.	45	M	Intestinal lymphangiectasia ?Filariasis	14·7	22	30·5		1·4	
V. Partial gastrectomy									
C.H.	63	M	Polya gastrectomy	13·1	341	50·7	Mean 54·6%	11·1	Mean 11·4%
K.M.	70	F	Polya gastrectomy	7·5	429	57·0		7·8	
P.W.	43	M	Polya gastrectomy	8·0	713	45·3		5·6	
W.G.	72	F	Polya gastrectomy	14·5	275	52·8		6·8	
S.B.	51	F	Polya gastrectomy	11·2	145	58·2		20·8	
C.Har.	47	M	Billroth I gastrectomy	2·3	381	63·5		16·5	

* Three-day collections of faeces only (mean value excluding the patients in whom faecal collections were incomplete).
† Intermittently treated with a gluten-free diet.
‡ On a 40 g fat diet.

and J.E.), and in the other three patients the plasma radioactivity was markedly diminished (Fig. 3). All these patients with obstructive jaundice had steatorrhoea, but there was no correlation between the degree of steatorrhoea and the malabsorption of the vitamin.

One patient, J.H., was studied again after choledochoduodenostomy, gastroenterostomy and enteroenterostomy had been performed to relieve the obstructive jaundice. The net absorption of α-tocopherol improved from 6·0% to 43·7% and, whereas before surgery no radioactivity was detected in the circulation, the plasma curve was now within normal range.

Three patients with chronic pancreatic insufficiency and steatorrhoea were studied. One of these patients (E.H.) had had a partial gastrectomy, and another a gastroenterostomy (E.K.).

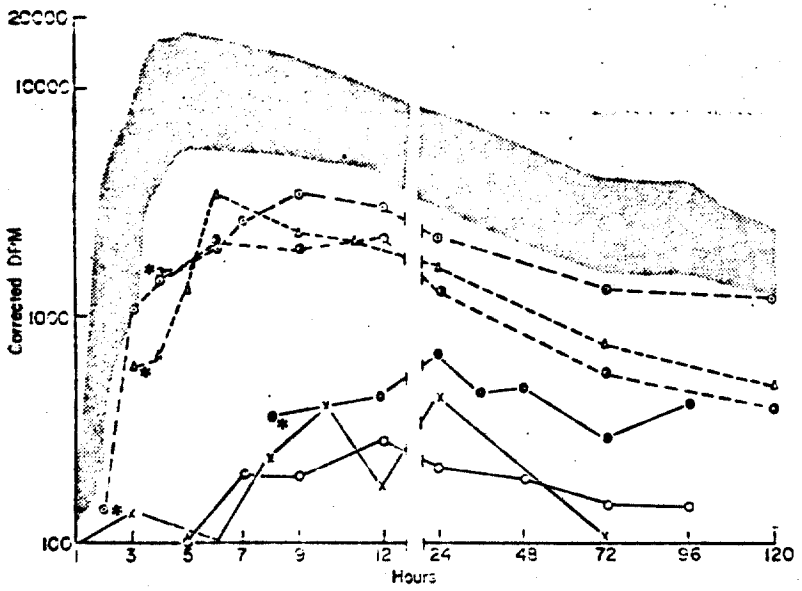

FIG. 3. Plasma radioactivity following an oral dose of [^{3}H]DL-α-tocopherol in three patients with biliary obstruction and three with chronic pancreatic exocrine insufficiency. In two subjects with biliary obstruction, no radioactivity was detected in plasma. O, D.T.; ○, E.Gw.; ×, E.Gar.; ●, C.P.; □, E.K.; △, E.H. * Less than 100 DPM/ml plasma up to this time. Shaded area represents range of plasma radioactivity in control subjects. —, Biliary obstruction; - - -, Pancreatic disease.

Net absorption was markedly diminished, ranging from 31·9% to 38·2% (mean 34·7%) of the ingested dose (Fig. 2). Plasma radioactivity in these three patients was also less than that in controls, although not as low as that found in patients with biliary obstruction (Fig. 3). The test was repeated in one patient (E.H.), with the contents of one capsule of Cotazym (Organon) added to the dose of vitamin E. On this occasion, absorption was normal.

Malabsorption in patients with mucosal abnormalities. The net absorption of α-tocopherol by five patients with untreated adult coeliac disease and one patient with tropical sprue ranged from 23·1% to 58·5% (mean 42·6%) (Fig. 2). Plasma radioactivity was at, or just below, the lower limit of the normal range, except in one patient (C.C.) who had very severe steatorrhoea and in whom it was impossible to detect any radioactivity in the plasma despite an apparent net absorption of 23·1%.

... group of patients, there was a good correlation between the failure to absorb vitamin ... and the degree of steatorrhoea ($P<0{\cdot}01$) (Fig. 4).

One patient (T.M.) with coeliac disease, who had taken a gluten-free diet intermittently, was also studied. He had virtually no steatorrhoea (faecal fat 7·1 g/day) and his plasma tocopherol level was normal. Although the curve of plasma radioactivity fell just below the normal range, this patient absorbed 84·6% of the dose.

Malabsorption in patients with intestinal lymphangiectasia. The two patients with intestinal lymphangiectasia absorbed only 28·5% and 30·5% of the dose of vitamin E (Fig. 2). The time of the appearance of the peak of radioactivity was delayed to 12 hr in one and to 10 hr in the other (Fig. 5). Neither patient had severe steatorrhoea (faecal fat excretions 13·8 g/day and 14·7 g/day respectively).

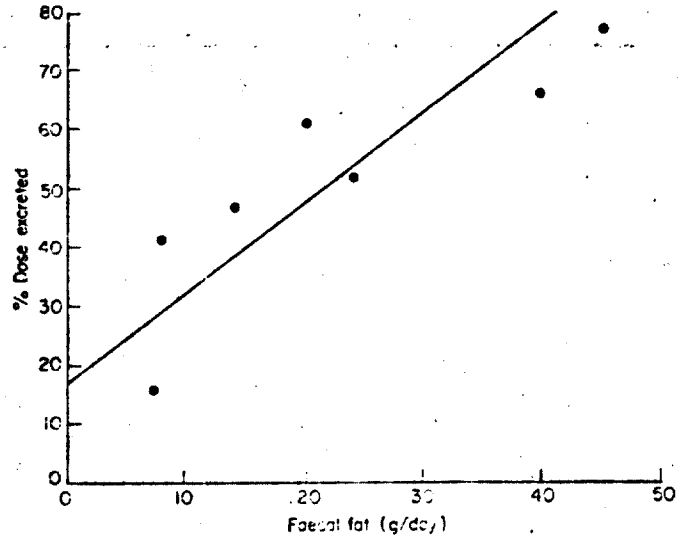

FIG. 4. Relationship between faecal excretion of fat and of radioactivity after an oral dose of [^{3}H]DL-α-tocopherol in seven patients with an abnormal small intestinal mucosa $P<0{\cdot}01$, $r = 0{\cdot}868$, $Y = 0{\cdot}653X-11$.

Malabsorption in patients after partial gastrectomy. The net absorption of vitamin E by seven patients who had had a partial gastrectomy was somewhat low with a mean value of 54·6% (range 45·3–63·5%) (Fig. 2). Radioactivity appeared earlier in the plasma of these patients than in that of control subjects, and in five out of seven there was significant activity in the plasma at 1 hr. Nevertheless, peak values occurred at the same time as in control subjects, and were just below or at the lower limit of the normal range.

Nature of radioactivity

Plasma radioactivity. In six experiments 79·7–100% (mean 90·3%) of the plasma radioactivity was recovered from the zone corresponding to vitamin E on thin-layer chromatography, and there was no concentration of radioactivity in other regions of the chromatogram. Plasma taken at both 5 hr and 24 hr after the dose had been given yielded similar results.

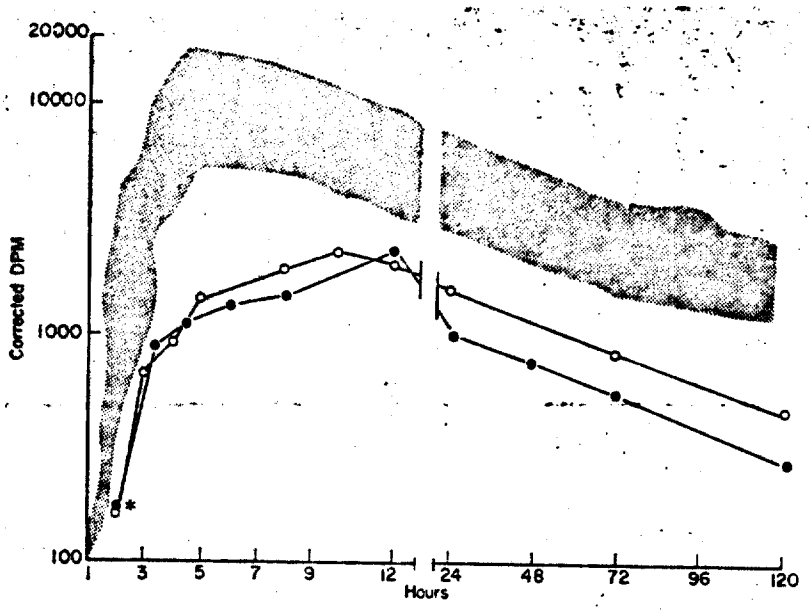

FIG. 5. Plasma radioactivity following an oral dose of [^{3}H]DL-α-tocopherol in two patients with intestinal lymphangiectasia. Shaded area represents range of plasma radioactivity in control subjects. * Less than 100 DPM/ml in plasma up to this time.

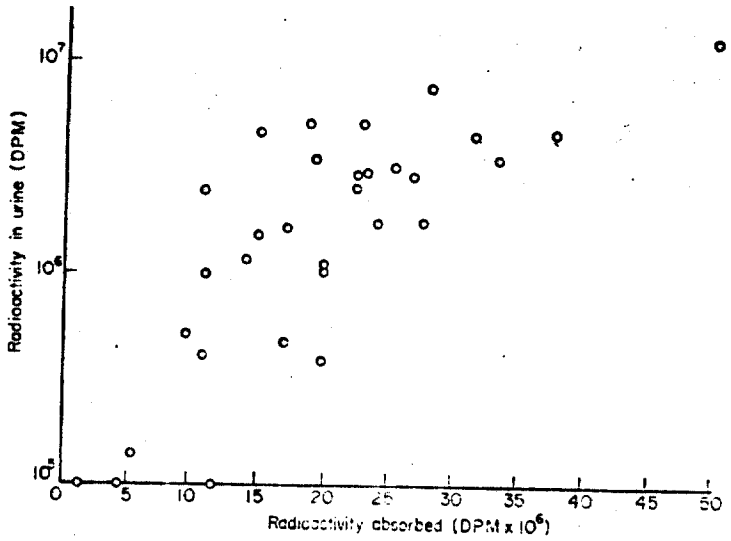

FIG. 6. Relationship between net absorption of [^{3}H]DL-α-tocopherol and radioactivity in urine. Urinary radioactivity is plotted on a logarithmic scale. $P<0.001$, $r = 0.6314$.

Faecal radioactivity. Two specimens of faeces from control subjects were examined by thin-layer chromatography after chloroform-methanol extraction. 3·3% and 3·9% of the faecal radioactivity were found in the methanol layer, and of the remainder, 26·7% and 32·6% had the same chromatographic mobility as α-tocopherol.

The nature of the radioactivity in the faeces of two subjects with malabsorption (J.W. with lymphangiectasia, E.A. with adult coeliac disease) was similarly studied. J.W. excreted 69·5% of the dose of radioactivity; 90·2% of this was lipid soluble, of which 75·4% had the same chromatographic mobility as α-tocopherol. E.A. excreted 52·2% of the dose; over 90% was lipid soluble, and of this, 41% behaved as α-tocopherol on thin-layer chromatography.

Urinary radioactivity. Less than 2% of the urinary radioactivity could be extracted into chloroform. After the evaporation of water from the urine, all the radioactivity remained in the residue. Radioactivity recovered in urine varied from 0·0% to 21·4% of the administered dose. The logarithm of the radioactivity excreted in the urine could be correlated with the net absorption ($r = 0·63$, $P < 0·001$) (Fig. 6).

DISCUSSION

Vitamin E deficiency is a common finding in patients with prolonged malabsorption (Binder *et al.*, 1965), but little is known about the mechanisms of absorption of this fat-soluble vitamin. Absorption in man has been measured previously using classical balance techniques. The intake of the vitamin has been assessed from food tables, and the excretion of vitamin E-like substances in the faeces has been measured by the Emmerie-Engel reaction (Emmerie & Engel, 1938) for reducing substances. In addition, the faecal excretion of vitamin E has been measured chemically after supplementing the diet with large doses of tocopherol acetate. With such doses, it is possible to measure the rise of the plasma concentration of the vitamin. The more recent availability of tritiated α-tocopherol has made it possible to study the absorption of physiological doses of vitamin E.

Normal subjects absorbed between 55·0% and 78·6% of an oral dose of 1 mg of tritiated vitamin E given in the way described. More than 90% of radioactivity recovered from the plasma at both 5 hr and 24 hr behaved as vitamin E on thin-layer chromatography using a single solvent system. After the absorptive phase was completed, the half-life of radioactivity in the plasma in normal subjects varied between 42 and 69 hr. A mean of 37% of the total urinary radioactivity was found in the first 24 hr. These findings may be compared with those of Schmandke & Schmid (1968) who found between 11·9 and 15·8 mg of tocopheronic acid in urine collected for 24 hr after the administration of 1 g of α-tocopherol to five control subjects. Although this represents only 1–1·5% of the dose, it is unlikely that tocopheronic acid is the only metabolite of vitamin E appearing in the urine. Simon *et al.* (1956) isolated tocopheronic acid and its γ-lactone from the urine of patients given 3·5 g of α-tocopherol by mouth, but were unable to quantitate the amount excreted. Further evidence in favour of the renal excretion of metabolites of vitamin E is given by the work of Stam[illegible] & Pascoe-Dawson (1959) who found that after the oral administration of [illegible]-tocopherol to rats, a mean of 37·9% of the administered radioactivity appeared in [illegible] in the first [illegible] hr.

The net absorption of small doses of [illegible] did not correlate well with the absorption of large doses of unlabelled vitamin E measured by balance techniques. Klatskin & Mylander (1952) found that normal subjects absorbed [illegible]% of a calculated daily intake of 33

α-tocopherol acetate (Roche) with 150 mg of vitamin E as α-tocopherol acetate. Schmid (1938) found a net absorption of 40% after 1 g of fat, but the excretion of α-tocopherol was measured in faeces collected for only 2 days.

There are several problems in comparing the results of the two methods. Firstly, although the biochemical method gives accurate recoveries of α-tocopherol added to faeces in vitro, it is not possible to ensure that there are no other reducing substances present capable of contributing to the Emmerie-Engel reaction. Secondly, we have shown that after a dose of [^{3}H]-α-tocopherol, between 15% and 70% of the faecal radioactivity is not α-tocopherol. Thus either vitamin E has been degraded during passage through the intestine, or it has been absorbed, metabolized and re-excreted into the gut. The third difficulty is the unreliability of estimates of dietary intake as measured by food tables. This may explain the apparently poor absorption of the vitamin by control subjects, and the improvement when the diet is supplemented with α-tocopherol, although it may be that tocopherol acetate is better absorbed than dietary vitamin E.

The present study was confined to an examination of the absorption of small doses of the pure vitamin as an alcohol. Patients with a variety of gastrointestinal diseases were studied in order to find out conditions in which vitamin E deficiency was most likely to occur and also to investigate the pathway of absorption. The most severe malabsorption was found in patients with biliary obstruction, suggesting that bile salts are necessary for the absorption of vitamin E, presumably because of their role in micelle formation. There was no relationship between the malabsorption of α-tocopherol and the degree of steatorrhoea in patients with biliary obstruction. This may be due in part to anorexia limiting the fat intake of the patients who were studied, although one patient (G.J.R.) had severe malabsorption of vitamin E and only mild steatorrhoea, even though he was eating a full ward diet containing 70 g of fat a day. On the other hand, patients with chronic pancreatic insufficiency had less severe malabsorption of the vitamin, presumably because of the presence of bile salt micelles.

Tritiated α-tocopherol was also absorbed poorly by patients with intestinal mucosal abnormalities, but in contrast to the group of patients with biliary disease there was a good correlation between the malabsorption of α-tocopherol and the degree of steatorrhoea. The net absorption of α-tocopherol was normal in only one patient with an abnormal jejunal mucosa (T.M.). This patient had taken a gluten-free diet intermittently for some time and had only minimal steatorrhoea.

Abnormalities of intestinal lymphatics produced quite severe malabsorption of vitamin E but very little steatorrhoea. The relatively severe malabsorption by patients with intestinal lymphangiectasia suggests that the lymphatic pathway is important in the absorption of the fat-soluble vitamin, and although Blomstrand & Borgstrom (1968) could recover only 20% of an oral dose of tritiated α-tocopherol from the thoracic duct of a patient, they did not measure the faecal excretion of the vitamin. It seems possible therefore, that much of the vitamin remained unabsorbed. On the other hand, Hayton & Quarfordt (1968) have shown recently that the absorption of triglycerides [illegible] to inhibit chylomicron synthesis, which supports the view [illegible] Borgstrom (1965) that long-chain fats can be absorbed via the portal vein [illegible] possible that, under some circumstances [illegible] vitamin E may be absorbed via the portal vein.

[illegible] vitamin E deficiency occurs quite commonly in patients who have had a partial [illegible] (Leonard *et al.*, 1966), the absorption of α-tocopherol by such patients appears to [illegible] only slightly reduced. In the seven patients in this study there was no correlation between [illegible] concentration of plasma tocopherol and the absorption of vitamin E. It is difficult to [illegible] the meaning of these results, especially as it has been shown that the mean concentration of vitamin E in patients with peptic ulceration is significantly lower than in normal subjects (Leonard *et al.*, 1966).

Further work will be necessary to assess whether dietary vitamin E is as well absorbed as free α-tocopherol.

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We wish to thank Miss J. Collins for her invaluable technical assistance, and Dr G. R. Thompson for his advice during this study. We are grateful to the physicians and surgeons of the Hammersmith Hospital for allowing us to study patients under their care. Part of this work was carried out whilst one of us (M.M.) was in receipt of a grant from the Medical Research Council.

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Lymphatic and Portal Venous Transport of α-Tocopherol and Cholesterol

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Abstract. The absorption of ^{3}H-α-tocopherol was studied in rats after intraduodenal administration in micellar solutions. Net absorption from mixed micelles in lymph fistula rats was 66%, but only 42% appeared in lymph and 3% was excreted in urine. Administration of ^{3}H-α-tocopherol in taurocholate micelles reduced both the rate of absorption into lymph and the proportion carried in chylomicrons. In studies with lymph and bile duct cannulated rats, up to 8% of the radioactivity was excreted in bile, irrespective of whether the cannula was in the mesenteric or thoracic lymph duct. These findings suggested that radioactivity was being absorbed via the portal vein, which was confirmed by demonstrating a higher concentration in portal than in aortic plasma. Radioactivity in plasma partly consisted of polar metabolites, in contrast t lymph where over 90% was free α-tocopherol. In parall experiments with ^{14}C-cholesterol, significant amounts of radi activity were excreted in bile and the ratio of portal: aort radioactivity again suggested absorption via the portal route The majority of ^{14}C-cholesterol in portal plasma was free stero in contrast to lymph where it was mainly esterified. Thes results suggest that small amounts of both α-tocopherol an free cholesterol can be transported from the intestine via th portal vein, although under normal circumstances absorptio of both compounds occurs mainly via the lymphatic pathway

Key-words: Absorption, bile, lymph, micelles, rat.

Vitamin E (α-tocopherol) has a terpene-like molecular structure and a very low solubility in water. Deficiency of this fat-soluble vitamin is not uncommon in patients with diseases causing malabsorption of dietary lipids [1]. The quantitative absorption of α-tocopherol in healthy subjects and patients with various malabsorptive disorders has recently been described by MacMahon and Neale [2], but information about the actual mechanisms involved remains incomplete. However, work by others has suggested that absorption occurs via the lymphatic pathway, and mainly as the free vitamin [3, 4].

In a recent communication, MacMahon and Thompson [5] demonstrated that efficient uptake of ^{3}H-α-tocopherol by the intestinal mucosa was dependent on the presence of bile salts in a concentration sufficient to solubilise lipids into a micellar phase within the intestinal lumen. These studies also confirmed that α-tocopherol was chiefly transported from the intestine via the lymphatic pathway, although the appearance of significant amounts of radioactivity in the bile of lymph fistula rats with cannulated bile ducts suggested that the portal venous route might also be involved. This route plays a role in the absorption of fatty acids [6] and also, possibly, in the absorption of vitamin A [7] and vitamin D [8]. In contrast, the transport of cholesterol has always been regarded as occuring exclusively via the lymphatic pathway [9].

In the present paper, the mechanism and route of absorption of α-tocopherol have been studied in the rat and compared with cholesterol. The results suggest that under certain circumstances, a significant proportion of α-tocopherol may be absorbed via the portal vein and, furthermore, that cholesterol also may be transported to some extent by this route.

Methods

Materials

Cholesterol-4-^{14}C and dl α-tocopherol-5-methyl-^{3}H were obtained commercially[1]; the labelled cholester was purified before use by thin-layer chromatograph on silica gel G, using chloroform as the solvent. Th purity of the labelled α-tocopherol was assessed befor use, as previously described [2].

Unlabelled dl α-tocopherol, cholesterol, and olei acid were obtained 96–99% pure[2]. Glycerol-1-mono oleate was obtained 90% pure[3], the remainder bein mainly di- and triglyceride, and used without furthe purification. Sodium taurocholate[4], containing no fre bile salts detectable on thin-layer chromatography was also used without further purification.

Preparation of Micellar Solutions

Mixed micellar solutions were prepared in a manne similar to that previously described [10] so as to contai 9.6 μmoles of oleic acid, 4.8 μmoles of monoolein an 0.15 μmoles of either labelled α-tocopherol or choles terol per ml of 20 mM taurocholate. Micellar solution of 20 mM taurocholate were similarly prepared, so a to contain the same amount of labelled α-tocopher or cholesterol as the mixed micellar solution. Th purity of the labelled compounds was re-assessed aft their incorporation into micellar solutions. Thin-laye chromatography of taurocholate micelles containin ^{3}H-α-tocopherol showed that 90–93% of the radio activity had the same mobility as α-tocopherol, th main contaminant being an unidentified less pola

1 Radiochemical Centre, Amersham.
2 Sigma, London.
3 Calbiochem., London.
4 Maybridge, Tintagel, Cornwall.

compound; less than 1% stayed at the origin. Chromatography of labelled cholesterol dissolved in taurocholate micelles showed that over 98% of the radioactivity had the mobility of authentic cholesterol, with less than 2% remaining at the origin.

Operative Procedures

Adult male Wistar rats weighing 250–300 g were anaesthetised with intraperitoneal pentobarbitone. The main mesenteric lymphatic and the duodenum (mesenteric lymph fistula rats) and in addition the common bile duct (mesenteric lymph-bile fistula rats) were cannulated as described previously [5]. Any lymphatics which did not drain into the main mesenteric lymph duct were ruptured and then occluded by application of a quick-drying adhesive[5]. In some rats a cannula was inserted into the thoracic duct instead of the mesenteric lymphatic (thoracic-duct fistula rats). Postoperatively, all rats were kept in restraining cages with free access to 4.3% dextrose in 0.18% sodium chloride. In addition lymph-bile fistula rats were perfused intraduodenally with 0.15 M sodium chloride at a rate of 2 ml/h during the 48 h recovery period allowed before the start of each experiment.

Absorption Studies

The animals were allowed to drink freely until 30 min. before the start of the experiment when all fluids were stopped. The labelled lipids were then infused intraduodenally in 4 ml of either the mixed micellar or taurocholate micellar solution over a 40 min. period by a pump[6], the dose being flushed through with 0.5 ml of normal saline. The rats were allowed access to oral fluids from two hours after the start of the experiment, the duodenal infusion of normal saline to lymph-bile fistula rats being restarted one hour earlier.

Lymph was collected in heparinised containers for 24 h, unless the samples were for thin-layer chromatography or ultracentrifugation, when they were collected on ice into tubes containing EDTA. Urine and bile were also collected for 24 h. At the end of this period, the rats were killed. Radioactivity in the small and large intestine, liver, faeces, plasma, lymph, bile and urine was assayed as previously described [5].

Portal Vein Experiments

In some mesenteric lymph-bile fistula rats, the animals were anaesthetised at 80–90 min. after the start of the infusion with an intraperitoneal dose of pentobarbitone, sufficient to produce coma within 3 min. Laparotomy was then quickly performed, and blood withdrawn from the portal vein and the abdominal aorta in quick succession into heparinised syringes; the plasma was separated off after centrifugation. In addition, portal and aortic blood was obtained in a similar manner from rats in which only the bile duct and duodenum had been cannulated.

Radioactivity in Lymph, Plasma and Bile

The amount of radioactivity in lymph and plasma was determined after extraction with 20 vol. of chloroform: methanol 2:1, v/v [11]. After separating the phases with water, the chloroform extract was dried on a rotary evaporator and counted in 10 ml of Butyl PBD[7] [(2,4 butylphenyl)-5(4' biphenylyl)-1,3,4,-oxidiazole] 6 G/L toluene. Radioactivity in bile was assayed by adding 0.2 ml to 10 ml of a water-miscible scintillation fluid, consisting of Butyl PBD 8 g, naphthalene 80 g, ethoxyethanol 400 ml and toluene 600 ml.

The nature of radioactivity in lymph, plasma and bile was determined by thin-layer chromatography. Samples of lymph and plasma were initially extracted into 20 vol. of acetone: alcohol 1:1 v/v. After centrifugation, the supernatant was dried on a rotary evaporator and then redissolved in 0.2 ml ethanol, of which 0.1 ml was applied to a thin-layer plate of silica gel G. For α-tocopherol, the plates were first slurried with fluorescein, and the samples co-chromatographed with 0.125 mg α-tocopherol and 0.25 mg of the anti-oxidant butylated hydroxytoluene (BHT). The plates were developed in 10% acetone/hexane (v/v) or in chloroform, under nitrogen and in darkness. Samples of 0.1 ml of bile were chromatographed in a similar manner but without prior extraction. After development, the plates were dried and the α-tocopherol zone located under ultraviolet light. The silica gel was divided into ten equal 2 cm strips, each of which was then scraped into 10 ml water-soluble scintillation fluid. The scintillation vials were shaken vigorously for one minute and then counted over a prolonged period in darkness.

The nature of radioactivity in experiments with ^{14}C-cholesterol was similarly investigated in lymph and plasma, but without the addition of carrier. After development of the plate in chloroform, the marker strips for free and esterified cholesterol were sprayed with 30% sulphuric acid and heated. The masked remainder of the plate was divided into 2 cm strips and the silica gel scraped into vials, and counted in water-soluble scintillation fluid. The nature of radioactivity in bile was investigated by thin-layer chromatography, as described by Hofmann [12].

Carriage in Lymph

The proportion of radioactivity carried in lymph chylomicrons after administration of ^{3}H-α-tocopherol was determined by ultracentrifugation. Samples of 0.5 ml of lymph were layered under 8.5 ml of 0.15 M sodium chloride and centrifuged at 4° for 1 h at

5 Eastman 910 adhesive, Liverpool, England.
6 Harvard Instrument Co., Millis, Mass., U.S.A.
7 Ciba, Duxford, Cambs.

Table 1. *Distribution of radioactivity after intraduodenal infusion of mixed micelles containing ^{3}H-α-tocopherol to mesenteric lymph fistula and lymph-bile fistula rats*

Preparation	Percentage administered radioactivity[a]					
	Lymph	Faeces	Bile	Urine	Liver	Recovery
Mesenteric lymph fistula (n = 7)	42.1 (39.4 – 46.3)	33.9 (27.2 – 37.4)	—	3.3 (1.3 – 4.1)	0.2 (0.1 – 0.8)	80.6 (72.5 – 85.8)
Mesenteric lymph-bile fistula (n = 4)	19.8 (16.4 – 23.9)	54.8 (48.3 – 59.5)	8.0 (7.4 – 10.0)	2.3 (2.1 – 2.5)	0.2 (0.1 – 0.30)	86.0 (81.9 – 89.9)

[a] Mean and range.
n = number of rats.

Table 2. *Distribution of radioactivity after intraduodenal infusion of taurocholate micelles containing ^{3}H-α-tocopherol to mesenteric lymph-bile fistula rats and thoracic duct-bile fistula rats*

Preparation	Percentage of administered radioactivity[a]					
	Lymph	Faeces	Bile	Urine	Liver	Recovery
Mesenteric lymph-bile fistula (n = 4)	13.3 (11.1 – 15.2)	51.6 (38.4 – 59.9)	9.2 (7.4 – 12.4)	3.6 (3.0 – 4.3)	0.2 (0.1 – 0.3)	77.9 (71.6 – 87.3)
Thoracic duct-bile fistula (n = 2)	6.4 (4.4 – 8.5)	54.7 (54.2 – 55.2)	4.2 (3.3 – 5.0)	1.4 (1.3 – 1.6)	0.2 (0.1 – 0.3)	67.8 (64.8 – 70.8)

[a] Mean and range.
n = number of rats.

100000 × *g*, in an MSE Superspeed 40 ultracentrifuge. The chylomicrons were aspirated from the surface of the tubes with a needle and syringe and counted directly in 10 ml of water-soluble scintillation fluid.

Results

Tocopherol

Absorption of ^{3}H-α-Tocopherol from a Mixed Micellar Solution. The absorption and distribution of radioactivity after infusing mixed micelles containing ^{3}H-α-tocopherol into the duodenum of mesenteric lymph fistula rats is shown in Table 1. A mean of 42.1% of the dose was recovered from the lymph and 33.9% from the faeces plus colonic contents in 24 h. In addition, 3.3% appeared in the urine and 0.2% in the liver, indicating that some radioactivity had been absorbed into plasma, despite cannulation of the main mesenteric lymphatic. This was confirmed by experiments with lymph-bile fistula rats, in which 8.0% of the administered dose was excreted in bile. Table 1 also shows that both the lymphatic absorption and the net absorption (as reflected by faecal radioactivity) of ^{3}H-α-tocopherol was considerably less in lymph-bile fistula rats than in rats with a simple lymph fistula.

Absorption of ^{3}H-α-Tocopherol from Bile Salt Micelles. To study the absorption of α-tocopherol under conditions where fatty acid absorption and chylomicron formation would be minimised, the labelled vitamin was administered to mesenteric lymph-bile fistula rats in 20 mM taurocholate micelles. The results (Table 2) show that the lymphatic absorption from taurocholate micelles was apparently lower (13.3% than in the rats given ^{3}H-α-tocopherol in mixed micelles (19.8%; see Table 1), whereas the net absorption and the amount of radioactivity in bile and urine were similar. However, the overall recovery was lower in rats given taurocholate micelles.

The absorption of α-tocopherol from taurocholate micelles was also measured in rats with a thoracic duct-bile fistula, to determine whether this gave a better recovery of lymph radioactivity than the mesenteric lymph fistula. In the two rats studied thoracic duct lymph radioactivity was less than half the amount found in mesenteric lymphatic cannulated rats, although faecal radioactivity was only slightly increased. Biliary and urinary radioactivity was also lower than in the mesenteric lymph fistula experiments, as was the overall recovery. These results did not indicate that lymphatic absorption of α-tocopherol would have been more accurately assessed by thoracic duct cannulation.

Transport in Lymph. Thin-layer chromatography of lipid extracts of lymph from 4 rats given mixed micelles showed that 94.2% of the radioactivity behaved as α-tocopherol, while in 5 rats given taurocholate micelles, the figure was 96.8%.

The effect of exogenous lipid on the rate and mode of transport of α-tocopherol in lymph was also assessed. Both the rate and extent of absorption into the lymph of lymph fistula rats was found to be increased when ^{3}H-α-tocopherol was administered in

Table 3. *Percentage of lymph radioactivity in chylomicrons after intraduodenal infusion of ^{3}H-α-tocopherol to lymph fistula rats*

Type of micellar solution	Percentage of lymph radioactivity in chylomicrons[a]		
	0—1 h	1—2 h	2—3 h
Mixed micelles (n = 3)	63.0 (56.3—67.1)	39.8 (38.0—42.0)	20.1 (18.7—20.9)
Taurocholate micelles (n = 3)	17.8 (14.5—21.7)	15.3 (14.3—16.7)	16.4 (14.7—17.8)

[a] Mean and range.
n = number of rats.

Table 4. *Radioactivity in portal venous and aortic plasma, 80 to 90 min after intraduodenal infusion of ^{3}H-α-tocopherol in taurocholate micelles to lymph-bile fistula rats*

Rat	dpm/ml plasma		Portal Aortic ratio
	Portal	Aortic	
1	822	436	1.89
2	932	470	1.98
3	542	393	1.38
4	5940[a]	5116[a]	1.16

[a] High concentration of radioactivity suggests a lymphatic-venous anastomosis.

mixed micelles compared to taurocholate micelles, as illustrated in Fig. 1. The proportion of the ^{3}H-α-tocopherol in lymph which was carried in chylomicrons is shown in Table 3. During the first hour after infusing mixed micelles, when the rate of triglyceride absorption is maximal, 63% of the α-tocopherol in lymph was in chylomicrons. This fell to 39.8% and to 20.1% during the second and third hours respectively. In contrast, when α-tocopherol was given in taurocholate micelles, only 17.8% was present in chylomicrons during the first hour, and even less during the subsequent two hours.

Radioactivity in Bile. Chloroform: methanol extraction of bile from two mesenteric lymph-bile fistula rats given ^{3}H-α-tocopherol showed that 81.6% and 90.4% of the radioactivity was soluble in aqueous methanol. Thin-layer chromatography of the chloroform soluble fraction showed that most of the radioactivity remained at the origin, less than 2% having the mobility of α-tocopherol.

Radioactivity in Portal and Aortic Plasma. Four lymph-bile fistula rats were given 10–20 μc of ^{3}H-α-tocopherol and the concentration of chloroform soluble radioactivity in chloroform: methanol extracts of portal venous plasma was compared with that in arterial plasma (Table 4). In each animal there was more radioactivity in portal than in arterial plasma at 80–90 min after the dose, which was reflected in the mean portal: arterial ratio of 1.6. During this period 0.8–2.3% of the administered dose had appeared in

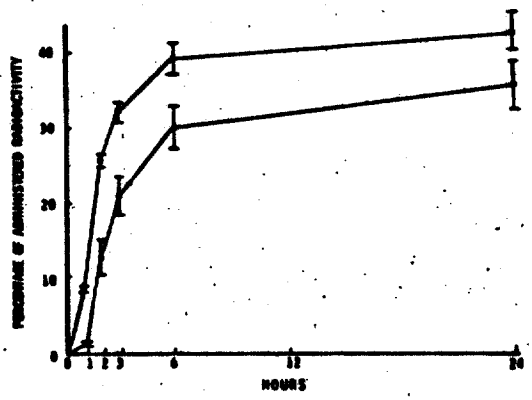

Fig. 1. Comparison of rate of absorption of ^{3}H-α-tocopherol into lymph after administration in taurocholate micelles to 5 rats (○) or in mixed micelles to 10 rats (●) with mesenteric lymph fistulae. — The graph shows the cumulative absorption of radioactivity into lymph, expressed as the mean ± 1 S.D.

Table 5. *Radioactivity in portal and aortic plasma at 80 to 90 min after intraduodenal administration of ^{3}H-α-tocopherol in taurocholate micelles to lymph-bile fistula rats*

Rat	dpm/ml plasma				Portal Aortic ^{3}H-α-tocopherol ratio
	Portal		Aortic		
	Total ^{3}H	^{3}H-α-tocopherol[a]	Total ^{3}H	^{3}H-α-tocopherol[a]	
5	3373	1221	2410	983	1.24
6	3576	2181	3288	2302	0.95
7	3078	1046	1826	803	1.30

[a] Radioactivity behaving chromatographically as α-tocopherol.

the lymph and 0.24–0.86% in the bile. In order to assess the nature of total plasma radioactivity, plasma was collected from 3 further rats and chromatographed after acetone: alcohol extraction (Table 5). Again, the overall concentration of radioactivity in portal plasma was consistently higher than in arterial, although on thin-layer chromatography only 34.0–70.0% behaved as α-tocopherol, most of the remainder being in more polar, unidentified compounds. The mean portal: arterial ratio of radioactivity having the Rf of α-tocopherol was 1.2, although in one rat it was less than unity.

Cholesterol

Absorption of Labelled Cholesterol in Lymph-Bile Fistula Rats. The absorption and recovery of ^{14}C-cholesterol at 24 h after intraduodenal administration to mesenteric lymph-bile fistula rats in micellar solutions is shown in Table 6. Absorption into lymph was equally low from mixed micelles and from taurocholate micelles, but in each instance a significant amount of radioactivity was excreted in bile. Similar amounts of

Table 6. *Distribution of radioactivity after intraduodenal infusion of ^{14}C-cholesterol in mixed or taurocholate micellar solution to mesenteric or thoracic duct lymph-bile fistula rats*

Type of micellar solution	Percentage administered radioactivity[a]				
	Lymph	Faeces	Bile	Small intestinal wall	Total recovery
Mixed micelles[b] (n = 3)	4.5 (3.7—5.8)	69.4 (63.9—78.6)	2.8 (2.3—3.2)	3.7 (1.3—6.1)	82.1 (76.8—86.9)
Taurocholate micelles[b] (n = 5)	3.7 (1.8—9.8)	60.0 (50.3—68.1)	2.6 (1.5—3.5)	6.0 (3.0—12.0)	73.4 (66.3—82.2)
Taurocholate micelles[c] (n = 3)	5.7 (3.2—8.7)	71.5 (66.8—79.4)	1.7 (1.2—2.4)	7.8 (1.4—12.1)	86.8 (75.9—94.3)

[a] Mean and range.
[b] Mesenteric lymph-bile fistula rats.
[c] Thoracic duct-bile fistula rats.
n = number of rats.

radioactivity also appeared in lymph and bile when ^{14}C-cholesterol was administered in taurocholate micelles to thoracic duct lymph-bile fistula rats (see Table 6).

Thin-layer chromatography of two samples of bile showed negligible radioactivity either at the origin or in the cholesterol zone. The majority of the radioactivity had the mobility of either taurocholate, or of taurodeoxycholate and taurochenodeoxycholate.

Nature of Radioactivity in Portal and Aortic Plasma, and in Lymph. At 80 to 90 min after administration of 5–30 μc labelled cholesterol to mesenteric lymph-bile fistula rats, consistently more radioactivity was in portal venous than in aortic arterial plasma, the portal: aortic ratio ranging from 1.32 to 3.45 (Table 7). During this time, 0.08–2.40% of the administered radioactivity appeared in lymph, and 0.07–0.39% in bile. In order to study the nature of this radioactivity, thin-layer chromatography was carried out on acetone: alcohol extracts of both portal plasma and lymph in three rats. At the time of sampling 22–75% of the radioactivity in portal blood was identified as cholesterol, the remainder being in more polar compounds; in lymph, over 98% of the radioactivity had the chromatographic mobility of cholesterol. In portal blood a mean of 69% of the labelled cholesterol was unesterified, whereas in lymph, 71% was esterified.

Two rats with a bile fistula but without a lymph fistula were also given intraduodenal taurocholate micelles containing labelled cholesterol. The percentage of the administered dose found in the portal and arterial plasma was, as would be expected, considerably greater than in the lymph fistula animals, and there was no concentration gradient. On thin-layer chromatography, 19–27% of the radioactivity was polar and remained at the origin, while the rest behaved as cholesterol. The majority of the labelled cholesterol in plasma was esterified, in contrast to the findings in lymph fistula rats, only 32–39% being present as the free sterol.

Table 7. *Radioactivity appearing in portal venous and aortic plasma[a] at 80 to 90 min after intraduodenal infusion of ^{14}C-cholesterol in taurocholate micelles to mesenteric lymph-bile fistula rats*

Rat	dpm/ml plasma		Portal / Aortic ratio
	Portal	Aortic	
1	752	218	3.45
2	1796	1362	1.32
3	387	263	1.47
4	691	474	1.46
5	349	240	1.45
6	122	87	1.40
7	1370	626	2.19

[a] Plasma of rats 1–4 extracted with chloroform: methanol (2/1 v/v) and of rats 5–7 with acetone: alcohol (1/1 v/v).

Discussion

The extent and route of absorption of a physiological dose (250 μg) of labelled α-tocopherol forms the main topic for discussion, although some unexpected findings in relation to cholesterol absorption also merit consideration. The net absorption of α-tocopherol, i.e. that percentage of the administered radioactivity not excreted in faeces within 24 h, averaged approximately 60% in lymph fistula rats given an intraduodenal dose of ^3H-α-tocopherol in a mixed micellar solution. This figure is similar to that observed by Krishnamurthy and Bieri [13], who administered the vitamin orally to rats, but is lower than the 80% absorption found by Sternberg and Pascoe-Dawson [14]. The percentage absorption of α-tocopherol in human subjects has been shown to be of the same order as in the rats used in the present study [2].

In the lymph fistula rats, just over two thirds of the net amount of α-tocopherol calculated to have been absorbed from the intestinal lumen appeared in lymph. This indicates that the lymphatic pathway is the main route of absorption in the intact animal, as

suggested by the experiments of Johnson and Pover [3], and subsequently confirmed by Kayden and Silber [4]. Blomstrand and Forsgren [15], however, recovered only 20% of an oral dose of labelled α-tocopherol in the lymph of a patient with a cannulated thoracic duct, but net absorption was not calculated. These authors found that the majority of α-tocopherol absorbed into lymph was unesterified, confirming the findings of Johnson and Pover [3], and those of the present study. In this respect, α-tocopherol behaves more like cholecalciferol (vitamin D) than cholesterol [10]. Ultracentrifugation of lymph showed that after administration in a mixed micellar solution α-tocopherol was mainly located in chylomicrons, particularly during the early phase of absorption. In contrast, when given in bile salt micelles, without exogenous lipid, less than 20% of the ^{3}H-α-tocopherol was in the chylomicron fraction of lymph. If the transport of α-tocopherol in lymph is governed in a similar manner to that of cholesterol, then it seems probable that most of the remainder was carried on very low density lipoproteins of intestinal origin [16]. The ability of mixed micellar lipid to enhance the rate of absorption of α-tocopherol into lymph is analogous to what has been shown in previous studies on the absorption of cholecalciferol [10].

Preliminary studies in lymph-fistula rats with an intact bile duct showed that 2–3% of the administered radioactivity was excreted in urine, a recognised excretory pathway for metabolites of α-tocopherol [17]. This suggested that some absorption must have taken place, in spite of the presence of a lymph fistula. The subsequent experiments were undertaken in order to determine whether this was due to incomplete cannulation of lymphatics draining the small intestine or whether absorption was occurring via another route. The extent to which absorbed radioactivity was bypassing the lymph cannula, in one way or another, can be gauged from the results of the studies in lymph-bile fistula rats. Although in these animals both the net absorption and the lymphatic absorption of α-tocopherol was much lower than in rats with a simple lymph fistula, nevertheless a significant amount of radioactivity was excreted in bile as well as in urine. The combined biliary and urinary radioactivity was equivalent to over 10% of the administered dose. It seemed unlikely that the site of cannulation of the lymph vessels could have been responsible because cannulating the thoracic duct, instead of the mesenteric lymphatic, did not increase the amount of radioactivity in lymph nor diminish the amounts appearing in bile and urine (see Table 2). Furthermore, when the lymphatic absorption of α-tocopherol was reduced by administration in taurocholate micelles instead of mixed micelles, no reduction in biliary or urinary radioactivity occurred, as would have been expected if lymphatic drainage was incomplete or if lymphatic-venous anastomoses were providing an important alternative route of absorption.

The results of subsequent studies in mesenteric lymph-bile fistula rats showed that at 80–90 min after a dose of labelled α-tocopherol there was a much higher concentration of counts in the portal vein than in the aorta (see Table 4). Further investigation of the nature of the radioactivity in plasma showed that only 34–70% behaved as α-tocopherol, the remainder consisting of unidentified, more polar compounds. This was in contrast to lymph where 94–97% of the radioactivity had the chromatographic mobility of α-tocopherol. Nevertheless, in 2 out of 3 rats (see Table 5) there was more ^{3}H-α-tocopherol in portal venous than aortic plasma. During the 80–90 min duration of these experiments an average of 2% of the administered ^{3}H was absorbed into lymph and a further 0.5% was excreted in bile. This ratio of radioactivity in lymph to that in bile is fairly similar to that found in the 24 h experiments. These findings suggest that some absorption of labelled α-tocopherol did occur via the portal vein and that this was a continuous process, as evidenced by biliary radioactivity.

In order to compare α-tocopherol with a compound which is said to be absorbed entirely by the lymphatic route, the absorption of cholesterol [9] was next studied in lymph-bile fistula rats. However, as with α-tocopherol, small but significant amounts of radioactivity were excreted in bile after administration of ^{14}C-cholesterol in either mixed or taurocholate micelles, irrespective of whether the mesenteric lymphatic or thoracic duct had been cannulated (see Table 6). Similarly, in short-term experiments the concentration of radioactivity was higher in portal venous than aortic plasma. By 90 min an average of 0.7% of the administered ^{14}C had appeared in lymph and 0.2% in bile, compared to 3.7% and 2.6% respectively in the 24 h experiments. The very low absorption of cholesterol into the lymph of rats with bile fistulae is presumably due to lack of the continuous supply of bile salts necessary for re-esterification of cholesterol within the intestinal mucosa [18]. On thin-layer chromatography 22–75% of the plasma radioactivity behaved as cholesterol, compared with 98% in lymph. It is of considerable interest that the majority of ^{14}C-cholesterol in lymph was esterified, whereas in plasma the majority was unesterified. In bile fistula rats without a lymph fistula, most of the labelled cholesterol found in plasma at 90 min was esterified. This makes it unlikely that the high proportion of free cholesterol in the plasma of lymph-bile fistula rats could be explained on the basis of rapid hydrolysis of absorbed cholesterol ester and its subsequent re-entry into the plasma as free cholesterol. It seems more likely that small amounts of free cholesterol were absorbed directly via the portal route, possibly because, in contrast to esterified cholesterol, the presence of a free hydroxyl group enabled it to undergo hydrogen bonding with water or plasma protein [19]. However, these experiments were not designed to detect net transfer of cholesterol and it is possible that

the results simply reflect exchange between labelled free cholesterol in the intestinal mucosa and unlabelled free cholesterol in portal plasma.

In conclusion, these studies show that ^{3}H-α-tocopherol is chiefly absorbed via the lymphatic pathway, without undergoing esterification. However, in both lymph-fistula and lymph-bile fistula rats there also appeared to be a significant amount of radioactivity undergoing absorption via the portal vein, only some of which was identified as α-tocopherol. The origin and nature of the large and variable amounts of portal venous radioactivity that was not α-tocopherol or cholesterol remains to be explained. Chemical transformation during incorporation into micellar solutions did occur, but only to a slight extent. The formation of metabolites of α-tocopherol within the intestinal lumen is probably the major source of polar radioactivity, although this seems a less likely explanation in the case of cholesterol. But in spite of the fact that much of the portal venous radioactivity was due to absorption of polar compounds, there was evidence of some transport of both α-tocopherol and free cholesterol via this pathway, under the experimental conditions described. Absorption of α-tocopherol via the portal pathway may explain the appearance of small amounts of the vitamin in the serum of patients with a β-lipoproteinaemia, in whom lymphatic transport of all lipids is absent [20]. It does not follow, however, that this route has any major quantitative significance in the absorption of cholesterol under normal circumstances.

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EFFECT OF HYPERVITAMINOSIS A ON THE TESTES OF THE RAT

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DEGENERATIVE lesions of the seminiferous tubules are among the most apparent morphological changes which accompany inanition. The proportion of tubules affected and the severity of the lesions are related to the degree of inanition and its duration. In this paper we report the occurrence of degenerative testicular lesions that are out of proportion to the nutritional states of rats receiving moderate doses of vitamin A and the inability of vitamin E to prevent these changes. During routine pathological examination of tissues [1] in an experiment on the toxic effects of long-continued administration of vitamin A, the degenerative changes in the testes were severe, while the weight gain and general condition of the animals were satisfactory. This observation plus the well-known effect of vitamin E deficiency in producing testicular lesions led to an investigation of the effect on the testicle of continued large doses of vitamin A and the influence of simultaneous administration of vitamin E.

EXPERIMENTAL

The experimental animals used were rats of Wistar strain 21 and 51 days of age. Diets employed were either Purina rat checkers or a synthetic diet previously used which gave good growth response curves. Its composition was as follows: casein (vitamin-free) 18%, dextrose 25%, cornstarch 45%, peanut oil 8%, $CaCO_3$ 1%, KH_2PO_4 2%, salt mixture [2] 1%.

The vitamin content per kilogram of diet was as follows: vitamin A 30,000 I. U. and vitamin D 5,000 I. U. (both incorporated into the peanut oil), thiamine hydrochloride 8 mg., pyridoxine hydrochloride 8 mg., riboflavin 8 mg., calcium pantothenate 16 mg., folic acid 5 mg., nicotinic acid 25 mg., paraaminobenzoic acid 300 mg., choline chloride 750 mg.

Vitamin A palmitate in sesame oil was given orally every morning, six days a weeks, at a dosage level of either 250 or 100 I. U. per gram of body weight. Vitamin E,[3] as the alpha tocopherol

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1. These tissues were supplied by the Hoffmann-LaRoche Company (Dr. R. Lewis), Nutley, N. J., from rats that had received 50 I. U. of vitamin A per gram of body weight for a period of one year and from dogs that had been given 25 I. U. per gram of body weight for a similar period.

2. Wolbach, S. B., and Maddock, C. L.: A. M. A. Arch. Path. 53:273, 1952.

3. The Hoffmann-LaRoche Company supplied the vitamin A and vitamin E.

acetate, was also given orally every afternoon, six days a week, at the dosage level of 10 or 100 mg. per 50 gm. of body weight. At the lower dosage this was diluted with peanut oil. The animals were weighed daily, and their daily food intake was recorded. They were bled by cardiac puncture under ether anesthesia, killed, and organ weights were obtained immediately on an analytical balance. Postmortem examinations were performed on all animals. Tissues were fixed in Zenker's fluid and stained by Mallory's methylene blue and eosin technic. The average diameters of seminiferous tubules were obtained by determining the widths of 10 or more tubules seen in cross section on a stained fixed section of the testicle by means of a mechanical stage equipped with a vernier scale. Variations in width on any one specimen never exceeded 0.02 mm. Testicular damage was graded as follows:

±—Tubules of normal size, a rare tubule showing loss of spermatogenesis; numbers of spermatozoa decreased

+—Tubules of normal size, occasional tubules showing loss of spermatogenesis: numbers of spermatozoa decreased [4]

++—Tubules slightly decreased in diameter; frequently depression of spermatogenesis visualized; occasional tubules entirely atrophic; occasional giant cells visualized

+++—Tubules decreased in diameter; occasional areas containing mature sperm in small numbers; most tubules atrophic and containing only Sertoli cells; many giant cells visualized

++++—Spermatogenesis absent; tubules small and containing only Sertoli cells and debris

TABLE 1.—*Relation of Testicular Weight to Body Weight in Fifty-Day-Old Rats*

No. of Animal	Vitamin A, I. U./Gm. Body Weight	Vitamin E, Mg./50 Gm. Body Weight	Final Weight, Gm.	Weight of Testicles, Gm.	Testicular Injury
9 VAE	0	..	96	1.301	0
2 VAE	250	..	90	0.533	+++
6 VAE	250	..	95	1.080	++
14 VAE	250	10	94	0.692	+++
18 VAE	250	100	96	0.528	+++

RESULTS

Group 1.—One group of animals, 20 in number, was maintained on the synthetic diet. Of these 20 animals, 16 were given vitamin A at the dosage level of 250 I. U. per gram of body weight. Of these 16 animals, 8 were also given vitamin E concomitantly, 4 at 10 mg. per 50 gm. of body weight and 4 at 100 mg. per 50 gm. of body weight. The remaining four animals were used as controls for the effect of vitamin E administration only, two at the level of 10 mg. per 50 gm. of body weight and two at the level of 100 mg. per 50 gm. of body weight.

Five animals of this group were killed at 50 days. This was done to estimate progress of the experiment and to study early changes, if any, in testicular injury. Of these animals, one received vitamin E only; two, vitamin A only; one vitamin A plus vitamin E at the level of 10 mg. per 50 gm. of body weight, and one, vitamin A plus vitamin E at the level of 100 mg. per 50 gm. of body weight. These five animals are listed in Table 1 and show some interesting relationships. Body weight in this

4. Leblond, C. P., and Clermont, Y.: Ann. New York Acad. Sc. **55**:545, 1952.

group of animals varied scarcely at all, ranging from 90 to 98 gm., with the control animal occupying an intermediate position. The testicular weights are, however, much less than those of the control, those of three animals representing one-half or less of the 1.3 gm. that the testicles of the control weighed.

The remainder of the animals in this group are listed in Table 2. Control animals on vitamin E grew well, were in excellent condition when killed, and showed no

TABLE 2.—*Testicular Injury in Rats Receiving Large Doses of Vitamin A and of Vitamins A and E (Synthetic Diet)*

No. of Animal	Vitamin A, I. U./Gm. Body Weight	Vitamin E, Mg./50 Gm. Body Weight	Age at Death, Days	Final Weight, Gm.	Weight of Testicles, Gm.	Average Diameter of Tubules, Mm.	Testicular Injury
10 VAE.....	0	10	50	134	1.404	0.20	+
11 VAE.....	0	100	63	161	2.140	0.24	0
12 VAE.....	0	100	63	188	2.706	0.25	0
Average.....	...	...	..	...	2.001	0.23	.....
1 VAE.....	250	0	50	85	1.414	0.20	++
3 VAE.....	250	0	63	131	1.939	0.21	+
4 VAE.....	250	0	62	121	1.916	0.23	+
5 VAE.....	250	0	63	104	1.880	0.21	+
7 VAE.....	250	0	56*	95	1.649	0.19	+
8 VAE.....	250	0	58*	137	1.983	0.24	±
Average.....	...	...	..	...	1.798	0.21	.....
13 VAE.....	250	10	63	108	0.692	0.14	+++
15 VAE.....	250	10	62*	92	0.796	0.16	+++
16 VAE.....	250	10	58*	147	2.218	0.24	0
17 VAE.....	250	100	59*	91	0.512	0.14	+++
19 VAE.....	250	100	62*	116	0.425	0.14	+++
20 VAE.....	250	100	62*	125	0.709	0.14	+++
Average.....	...	...	..	...	0.889	0.16	.....

* Died.

TABLE 3.—*Testicular Injury in Rats Receiving Large Doses of Vitamin A (Stock Diet)*

No. of Animal	Vitamin A, I. U./Gm. Body Weight	Vitamin E, Mg./50 Gm. Body Weight	Age at Death, Days	Final Weight, Gm.	Weight of Testicles, Gm.	Average Diameter of Tubules, Mm.	Testicular Injury
1 AK.....	250	0	88*	73	0.787	0.13	++++
2 AK.....	250	0	99†	110	1.446	0.17	++++
3 AK.....	250	0	114†	185	1.820	0.22	++++
4 AK.....	250	0	114†	205	1.205	0.18	++++
5 AK.....	250	0	114†	208	2.287	0.23	++++
6 AK.....	250	0	99†	128	1.174	0.20	++++

* Died.
† Killed.

pathologic changes in any of the tissues examined. Among those animals receiving only vitamin A, two died of hemorrhage; one was in poor nutritional state, and one showed transient signs of a hemorrhagic tendency, which was absent at the end of the experiment. This one, as well as the two remaining rats, was in reasonably good condition and would undoubtedly have lived for a longer period. Testicular injury ranged from ± in one, 1 + in four, to 2 + in one. The two 50-day animals in this category showed testicular changes of 2 + and 3 +.

In the combined vitamin A and E subgroup, there were five moribund animals showing multiple and massive hemorrhages. The sixth animal also showed hemorrhage in one hindleg but would probably have survived a few more days. One animal which died because of hemorrhage had normal testicles; the rest showed maximum or 3 + injury. The two 50-day animals in this group also showed 3 + injury. There appeared to be no relationship between the hemorrhagic syndrome and the testicular change.

Group 2.—Six weanling rats maintained on Purina rat checkers were started on doses of vitamin A at the level of 250 I. U. per gram of body weight. The results are given in Table 3. The first animal in this series died apparently of toxic effects of vitamin A administration and not of hemorrhage. Healed fractures of the tibiae

TABLE 4.—*Testicular Injury in Rats Receiving Large Doses of Vitamin A (Synthetic Diet)*

No. of Animal	Vitamin A, I. U./Gm. Body Weight	Vitamin E, Mg./50 Gm. Body Weight	Age at Death, Days	Final Weight, Gm.	Weight of Testicles, Gm.	Average Diameter of Tubules, Mm.	Testicular Injury
38 VAE.....	100	0	135*	173	2.464	0.29	++
39 VAE.....	100	10	100*	170	2.250	0.22	++
41 VAE.....	100	100	75*	140	2.525	0.24	++
42 VAE.....	100	100	75	176	2.353	0.23	++

* Died.

TABLE 5.—*Testicular Injury in Rats Receiving Large Doses of Vitamin A (Stock Diet)*

No. of Animal	Vitamin A, I. U./Gm. Body Weight	Vitamin E, Mg./50 Gm. Body Weight	Age at Death,* Days	Final Weight, Gm.	Weight of Testicles, Gm.	Average Diameter of Tubules, Mm.	Testicular Injury
49 VAE.....	100	0	95	272	2.920	0.27	0
51 VAE.....	100	10	103	251	3.109	0.23	+
53 VAE.....	100	10	95	338	3.423	0.28	+
54 VAE.....	100	10	93	372	3.567	0.24	+
55 VAE.....	100	100	95	325	2.920	0.28	+
56 VAE.....	100	100	93	267	2.918	0.23	+
57 VAE.....	100	100	93	347	2.904	0.27	+
58 VAE.....	100	100	93	352	3.004	0.27	+
59 VAE.....	100	100	60	256	2.294	0.26	+
60 VAE.....	100	100	103	311	2.846	0.25	+

* Killed.

were present. The other animals were in a state of poor to moderate nutrition, showing the bony changes (recent and healed fractures, the consequence of accelerated remodelling processes at the growing ends of bone [5]) characteristic of excessive vitamin A intake. The testicles were small to moderate in size, and all showed advanced degenerative changes.

Group 3.—Because of failure of most of the animals in the previous group to survive beyond 63 days (representing approximately 26 doses of vitamin A) on combined vitamins A and E at the vitamin A level of 250 I. U. per gram of body weight, vitamin A intake of this group was lowered to 100 I. U. per gram of body weight. Four animals on the synthetic diet were maintained on this regimen, and

5. Wolbach, S. B.: J. Bone & Joint Surg. **29**:171, 1947.

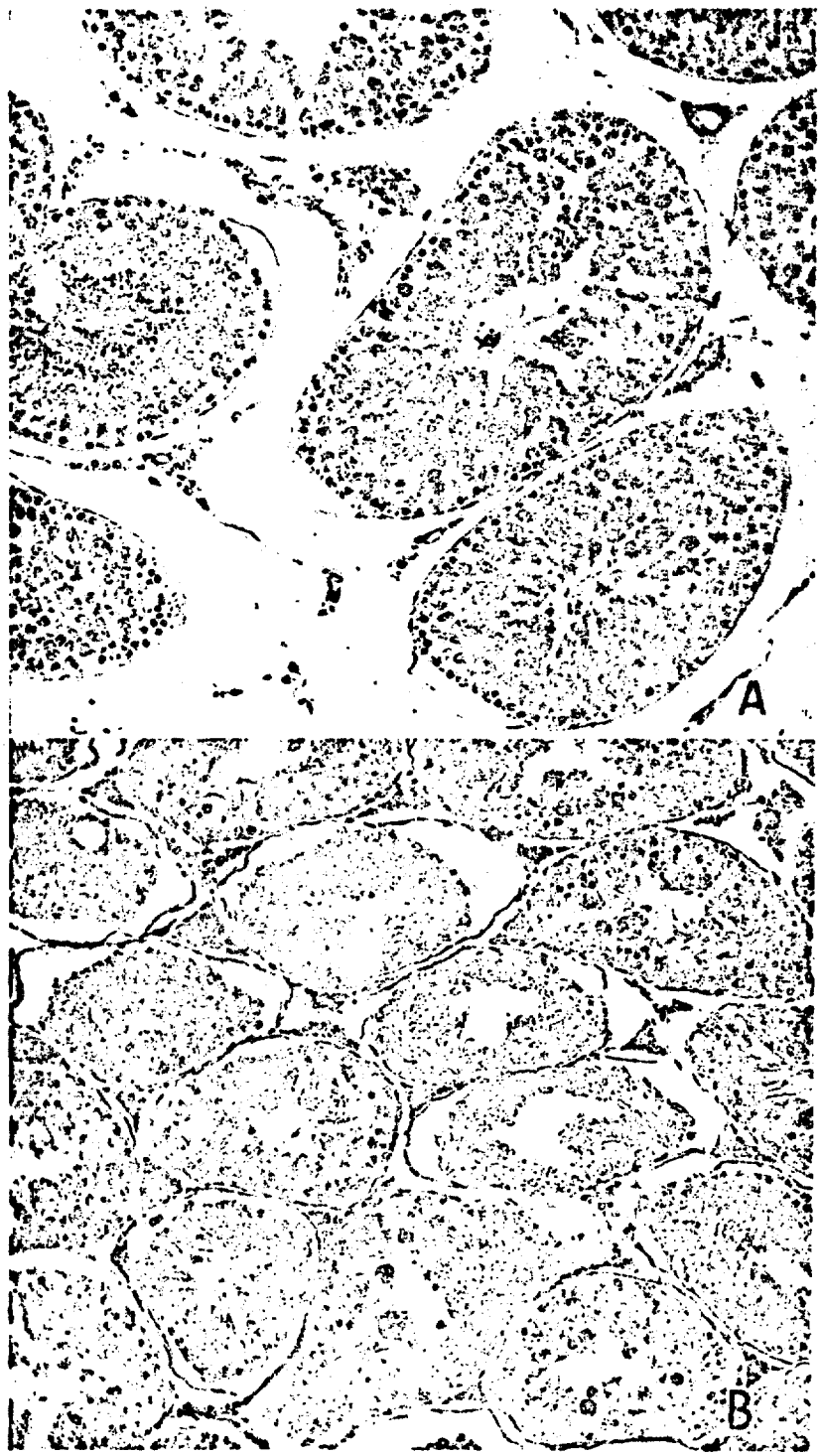

Fig. 1.—*A*, testis of 100-day-old rat raised on a synthetic diet and given vitamin A in 51 doses of 100 I. U. per gram of body weight and vitamin E at 10 mg. per 50 gm. of body weight starting at 21 days of age. There is clumping of the spermatids and spermatocytes and loss of chromatin of the spermatocytes. There is moderate edema between tubules; × 125. *B*, testes of 88-day-old rat raised on stock diet and given 44 doses of vitamin A at 250 I. U. per gram of body weight starting at the age of 27 days. The tubules are much smaller than in Figure 1*A*. They all show profound degenerative changes; × 125.

Fig. 2.—*A*, one tubule of same animal as in Figure 1*B*, showing details of giant cell formation and degeneration of spermatocytes; × 400. *B*, one tubule from a rat aged 99 days, raised on a stock diet, and given 54 doses of vitamin A at 250 I. U. per gram of body weight starting at age of 27 days. This shows the progressive degeneration of spermatocytes and spermatogonia; × 400.

survival was prolonged from 75 days to 135 days (the upper limit occurring in the non-vitamin-E-supplemented animal), but death from hemorrhage again supervened. Testicular change was designated as 2 + in all four animals. The results are given in Table 4.

Group 4.—Ten weanling rats on the stock diet were maintained on the vitamin A level of 100 I. U. per gram of body weight, three being given vitamin E at 10 mg. per 50 gm. of body weight and six at the 100 mg. per 50 gm. of body weight. All were killed at periods varying between 80 and 103 days, and all were in an excellent state of nutrition. The testicles showed no gross changes or diminution in size, but on histological examination all of them, with the exception of the one rat on vitamin A only, showed minimal testicular change. Results are given in Table 5.

Group 5.—Observations were made on the effect of long-maintained large doses of vitamin A on the mature rat to note the action of the vitamin upon the fully developed testicle. Five 51-day-old sexually mature rats that had been maintained on a stock diet since weaning were given vitamin A at two levels, three at 250 I. U. per gram of body weight and two at 100 I. U. per gram of body weight. One rat on each vitamin A dosage also received daily supplements of vitamin E, 100 mg. per 50 gm. of body weight. After a 100-day period on this regimen the rats were killed. No evidence of testicular injury could be demonstrated either in the straight vitamin A category or in that supplemented with vitamin E.

The histologic characteristics of the testicular degeneration in the early stage consisted of a diminution in the numbers of spermatozoa and a loss of chromatin by the spermatids and spermatocytes (Fig. 1*A*). Late lesions contained spermatocytes with bead-like nuclei with diminished chromatin, as described by Mason,[6] and in the more advanced lesions there were sloughing of the spermatocytes and clumping of their nuclei and the nuclei of spermatids to form giant cells (Figs. 1*B* and 2). At the end stage nearly complete sloughing of the germinal epithelium occurred. While the changes seen are thought to be nonspecific and to conform in general to those elicited in inanition, the chromolytic nuclear changes and the giant cell formation resemble the changes described in vitamin E deficiency. The reversibility of the lesion was not investigated.

COMMENT

It seems fairly clear from the data submitted and from evidence on hand from the long-continued toxicity experiments on vitamin A conducted in the Hoffmann-LaRoche Laboratories, as well as rather fragmentary observations made on previous animals subjected to the hypervitaminosis A regimen, that testicular injury is one of the results of the prolonged administration of large doses (50 to 250 I. U. per gram of body weight) of this vitamin. It would also appear that this effect is potentiated by the concurrent intake of vitamin E. An explanation of this enhancing effect of vitamin E may reside in its antioxidant action, which presumably occurred in the blood stream or tissues since the two vitamins were administered at different times. Our data are not sufficiently extensive or inclusive to assess what part, if any, diet plays in this picture, but there is an indication that on the synthetic diet the rats were more prone to development of the testicular lesions.

6. Mason, K. E.: Am. J. Anat. 52:153, 1933.

The complicating factor of hemorrhage, which apparently occurs earlier and with greater regularity in our rats on synthetic diet, makes it impossible to carry on the experiment for equally long periods of time at equivalent levels of vitamin A in the two diets used, synthetic and stock. One of the most interesting features of the study, one that will be reported in a subsequent paper, has been the intensification of the hemorrhagic diathesis induced in hypervitaminosis A by the simultaneous administration of vitamin E.

SUMMARY

Long-continued ingestion of moderate doses of vitamin A in weanling rats elicits degenerative lesions of the testis, but in mature rats receiving similar dosages these lesions do not develop.

Vitamin E given concurrently with vitamin A potentiates the degenerative change.

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Reticulocytosis in Response to Dietary Antioxidants

Abstract. *Alpha-tocopheryl acetate, 1,2,dihydro-6-ethoxy-2,2,4-trimethylquinoline, and butylated hydroxytoluene increased the number of circulating reticulocytes when added to the diet of chickens. Hematocrit values were not reduced and erythrocyte life-spans were not shortened by the antioxidants. The reticulocytosis is attributed to delayed loss of reticular material from the maturing erythrocytes.*

Reticulocytosis has been reported to occur in response to vitamin E and to coenzyme Q_4 chromanol treatment of vitamin E–deficient monkeys, human infants, and children (*1*). The experiments described here demonstrate that antioxidants, when fed to chicks kept under normal dietary conditions, retard the maturation of reticulocytes.

In two experiments chicks were fed balanced diets based on natural ingredients and supplemented with antioxidants as indicated in Table 1. Retic-

ulocytes were counted by the slide method as previously described (*2*) after the chicks had been on experiment for several weeks. Hematocrit values were also determined. In a third experiment the effect of dietary antioxidants on the life-span of erythrocytes was studied by labeling in vivo with selenomethionine ^{75}Se (*3*). This method estimates both production rate and survival time of erythrocytes. A different group of chicks was bled for each set of determinations of radioactivity in order to avoid the effect of blood loss on erythropoiesis which would result from repeated bleedings of the same birds. The hematocrit value of each sample was measured in order to relate radioactivity to packed cell volume. Reticulocytes were counted in the blood samples drawn 27 days after injection.

The reticulocyte counts of the chicks fed the control diets were similar to those previously reported for growing chicks (*2*). Supplementation of the diets with 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (EQ), butylated hydroxytoluene (BHT), and *dl*-alpha-tocopheryl acetate (TOC) increased reticulocyte counts significantly ($P < .01$) in all experiments. It should be noted that the levels of TOC fed were in excess of the requirement of growing chicks.

Hematocrit values were similar for the blood samples from the chicks fed the basal diets and the diets supplemented with antioxidants. It might accordingly be assumed that the observed reticulocytosis was not due to an insufficiency of red blood cells. This assumption was borne out by the results of the third experiment (Table 2) which indicate no acceleration of the rate at which red blood cells were either discharged into or removed from the circulation of the birds receiving antioxidants. Radioactivity of the fluid hemolysates from red blood cells of birds fed TOC was higher 27 days after injection of the tracer than in the case of the control birds, or the birds fed EQ. Although the markedly greater standard deviations of the values for the group treated with TOC make it difficult to calculate a valid estimate of the significance of the difference, it is apparent that erythrocyte turnover was slower in some of the birds treated with TOC.

It is emphasized that the reticulocytosis reported here occurred when antioxidants were fed to nutritionally normal animals in contrast to that reported in connection with alleviation of a vitamin E deficiency (*1*). In the present experiments the control chickens were not deficient in vitamin E and the antioxidants were administered for several weeks prior to counting of reticulocytes. The reticulocytosis is attributed to retardation of the maturation process of the cells rather than to acceleration of erythropoiesis. Reticulocytes contain more phospholipid than do mature cells (*4*), and there is other evidence that cell aging is accompanied by a decrease in total lipids, cholesterol, and phospholipids (*5*). Dietary antioxidants have been shown to moderate the adverse effects of exposure to hyperoxia (*6*), and could therefore be expected to retard autoxidation of tissue lipids under conditions of normal oxygen concentration.

If the disappearance of reticular material is related to the decline in lipids in maturing red blood cells and involves autoxidation of the lipids, delay of the maturation process could result from antioxidant protection of the lipid components.

Table 1. Effect of dietary antioxidants on reticulocyte count and hematocrit value. Results are given as means ± standard deviation. In experiment 1, eight male White Leghorn chicks per treatment were placed on test at 8 weeks of age; in experiment 2, ten male White Leghorn chicks were on each treatment from 0 to 4 weeks of age. BHT, butylated hydroxytoluene; EQ, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline; and TOC, *dl*-alpha-tocopheryl acetate.

Treatment	After 2 weeks	After 3 weeks	
	Reticulocytes (%)	Reticulocytes (%)	Hematocrit (%)
	Experiment 1		
Control	11.8 ± 2.0	13.8 ± 2.3	30.8 ± 2.1
BHT (0.5 g/kg)	11.9 ± 2.5	19.2 ± 5.5	30.9 ± 2.5
BHT (5.0 g/kg)	16.7 ± 3.8	19.3 ± 7.1	30.9 ± 2.3
EQ (0.5 g/kg)	18.5 ± 4.6	23.0 ± 5.3	30.1 ± 2.2
EQ (5.0 g/kg)	29.6 ± 6.9	46.7 ± 12.0	28.3 ± 3.3
TOC (120 unit/kg)	20.7 ± 3.3	31.6 ± 6.8	30.5 ± 3.1
	Experiment 2		
Control	12.5 ± 2.1		28.0 ± 1.9
EQ (0.5 g/kg)	16.3 ± 2.4		27.8 ± 4.7
EQ (5.0 g/kg)	18.6 ± 4.6		27.3 ± 5.7
TOC (220 unit/kg)	18.7 ± 2.0		29.8 ± 2.1

Table 2. Erythrocyte turnover in chicks fed antioxidants. Male White Leghorn chicks were fed a balanced diet supplemented as indicated from 0 to 7 weeks of age. They were then injected intravenously with 1 μc of ^{75}Se-labeled L-selenomethionine. Their red cells were tested 2, 9, and 27 days later for ^{75}Se (count/min), and the percentage of reticulocytes was determined on day 27. Results are average counts per minute ± standard deviation of fluid lysates from 1 ml of packed cells. EQ, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline; TOC, *dl*-alpha-tocopheryl acetate.

Treatment	Turnover after injection			
	Day 2* (count/min)	Day 9† (count/min)	Day 27†	
			(count/min)	Reticulocytes (%)
Control	302 ± 63	926 ± 194	579 ± 61	11.8 ± 2.5
EQ (2.5 g/kg)	252 ± 119	949 ± 269	554 ± 96	16.9 ± 3.0
TOC (220 unit/kg)	308 ± 95	887 ± 316	784 ± 243	18.6 ± 5.1

* Twenty chicks per treatment. † Ten chicks per treatment.

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EFFECTS OF THE ADMINISTRATION OF A VITAMIN E CONCENTRATE AND OF CHOLESTEROL AND BILE SALT ON THE AORTA OF THE RAT

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LOS ANGELES

THE influence of vitamin E (alpha tocopherol) on cholesterol metabolism and atherosclerosis is still obscure, and reports in the literature are contradictory. According to Monnier and associates,[1] hypervitaminosis-E had no significant effect on total blood cholesterol in the rat, and Dam [2] observed that *d,l*-tocopherol acetate did not modify the aortic cholesterol deposition of rabbits or chicks fed diets high in cholesterol. Morgulis and Spencer,[3] on the other hand, reported a significant rise of blood cholesterol in rabbits fed a vitamin E–deficient diet, while Bruger [4] found that vitamin E markedly increased the cholesterol content of the aorta of the rabbit fed a high cholesterol diet.

In view of these contradictory results the relation of vitamin E to cholesterol metabolism was investigated. The present report deals with the effects of avitaminosis-E and those of high doses of a vitamin E concentrate on cholesterol metabolism and atherosclerosis in the rat.

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Contribution no. 195 from the Department of Biochemistry and Nutrition, University of Southern California.

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EXPERIMENTAL PROCEDURES

Nursing rats were restricted to a diet deficient in vitamin E.[5] The young rats were fed the same diet until, a few days after weaning, the litters were divided into four experimental groups of 4 males and 4 females each, as shown in the table. Litter mates were distributed evenly as far as possible.

Groups 1 and 2 were fed the deficient diet without supplement (E-deficient). For groups 3 and 4 (E-high) the diet was supplemented once weekly with 150 mg. of a vitamin E concentrate prepared from vegetable oils and containing 34 per cent "mixed tocopherols"[6]; the concentrate was fed with a syringe, after being diluted with ethyl laurate. Somewhat older rats fed this laboratory's stock diet served as controls (groups 5 and 6). For groups 2, 4 and 6 (E-deficient, C + B) cholesterol[7] was added to the diet at a level of 1 per cent, and, in order to increase absorption of cholesterol, 0.5 per cent of bile salt was added.

Body weights were noted during the experimental periods, which varied from twenty to twenty-seven weeks. At autopsy total cholesterol levels were determined for blood and various tissues, a modified Schoenheimer-Sperry-Chaney procedure being used.[10]

The aorta was cut open lengthwise and fixed in formaldehyde solution. Sample strips, especially of the thoracic portion, were embedded in gelatin and examined as frozen sections stained with nile blue sulfate. Interesting specimens were also stained with other dyes, gelatin and paraffin sections being used.[11]

Cholesterol was identified by its birefringence with the help of a pair of polaroid films[12] inserted in the microscope. To distinguish small cholesterol deposits from other anisotropic material, such as collagenous fibers, the slides were examined before and after extraction with chloroform. Pairs of photomicrographs were also made in this manner. The work was rendered difficult because, on storage, fixed tissues and sections slowly lose by diffusion part of their stainable fat and cholesterol.

RESULTS

Body Weight.—At an age of approximately 80 days, the weight curves of the vitamin E–deficient animals began to level off. During the following month the male rats of group 1 (E-deficient) lost an average of about 40 Gm. and were in such poor general condition that they were killed and examined. At that time, respiratory infections and lesions of the skin were widespread, but

5. The diet was made up as follows: 1,200 Gm. of sugar; 800 Gm. of starch; 460 Gm. of lard; 800 Gm. of a commercial casein; 320 Gm. of yeast (strain G); 300 Gm. of cellulose flour (celluflour[8]); 120 Gm. of salt mix; 1.2 Gm. of a vitamin D (calciferol) concentrate (drisdol[9]); 1 mg. of carotene.

6. The concentrate is marketed by Distillation Products, Inc.

7. Dr. F. Fenger, of the Armour Laboratories, and Mr. Noble F. Payton, of the Suburban Chemical Company, Chicago, supplied some of the cholesterol used.

8. Schoenheimer, R.: Biochem. Ztschr. **147**:258, 1924.

9. Extract of ox bile U. S. P.; both Wilson's and Armour's products were used; Dr. F. Fenger of the Armour Laboratories, supplied some of the bile salt used.

10. A description of this modification of the Schoenheimer-Sperry-Chaney procedure is to be published.

11. The Department of Anatomy of the University of Southern California School of Medicine lent two microtomes for our use.

12. Polaroid films are manufactured by the Polaroid Company, Cambridge, Mass.

only 1 rat exhibited muscular symptoms. The animals of group 3 (E-high) grew at a normal rate.

The influence on body weight of dietary cholesterol and bile salt was not uniform. A beneficial effect was observed in the male rats of group 2 (E-deficient, C + B). The female animals of this group, however, gained significantly less than their litter mates on the same diet without cholesterol and bile salt. In group 4 (E-high, C + B) the male rats fed the high cholesterol diet gained significantly less weight than their respective litter mates, whose diet was not supplemented with cholesterol and bile salt. The female rats of this group, however, were not much affected.

Sex Organs.—The rats of group 1 (E-deficient) showed the expected genital symptoms. Addition of cholesterol and bile salt to the diet (group 2) aggravated these defects. The male animals of group 3 (E-high) also appeared

Effect of Vitamin E Deficiency as Influenced by the Administration of a Vitamin E Concentrate, Cholesterol and Bile Salt

(Feeding Periods: 20 to 27 Weeks)

Group	Vitamin E Concentrate, Mg. per Wk.	Dietary Cholesterol + Bile Salt	Rats	Plasma Cholesterol, Mg. per 100 Cc.	Liver Cholesterol, per Cent	Histologic Changes of Aorta—Rats Showing Given Change		
						Fatty Infiltration	Cholesterol Deposits	Intimal Sclerosis
1	None	None	7	41 ± 4	0.25 ± 0.02	7	0	0
2	None	C + B	5	155 ± 12	2.55 ± 0.61	5	0	0
3	150	None	7	56 ± 2	0.30 ± 0.01	5	7	6
4	150	C + B	7	122 ± 11	3.68 ± 0.00	4	0	0
5	Stock diet	None	17(7)*	60 ± 3	0.21 ± 0.02	3	0	0
6	Stock diet	C + B	5(8)*	128 ± 12	2.03 ± 0.64	4	0	2

* The number in parentheses is the number of aortas examined histologically. The animals of groups 5 and 6 were 1 to 2 years old. Group 6 includes 3 rats fed the experimental diet for thirty-eight weeks. Further control rats, from other experiments, were not included in this table, because conditions were slightly different. The aortas of those animals showed the same histologic picture.

sterile, while those of group 4 (E-high, C + B) appeared fertile but had smaller prostates and seminal vesicles.

Liver.—As was to be expected,[13] the livers of the animals fed cholesterol and bile salt (groups 2, 4 and 6) were pale and enlarged, regardless of the level of the vitamin E intake.

Tissue Cholesterol Levels.—Total cholesterol was determined for plasma, liver, adrenal glands, kidneys and spleen. As a consequence of the high cholesterol intake, cholesterol was increased in the plasma about two to four fold, in the liver approximately five to ten fold (table 1), and in the adrenal glands about 1.5 to 3 times. The level of vitamin E in the diet did not influence these changes significantly. The cholesterol contents of the kidneys and the spleen were normal except in group 2 (E-deficient, C + B), in which they were increased by about 50 to 100 per cent.

Morphology of Aorta.—Several of the vitamin E-deficient rats (groups 1 and 2, table) had white fatty plaques in the aorta. On histologic examination,

13. Anitschkow, N., and Chalatow, S. S., quoted and confirmed by Wacker, L., and Hueck, W.: Arch. f. exper. Path. u. Pharmakol. **74**:416, 1913.

all aortas of these groups had fatty films on endothelium and elastic lamellas and had more stainable fat and foam cells in the endothelium than were found in control material. The foam cells were not birefringent, a species characteristic of rats and mice, as emphasized by Leffkowitz and Rosenberg.[14] The fat imbibition appeared equally high in groups 1 (E-deficient) and 2 (E-deficient, C + B). Sparse cholesterol crystals were seen in the adventitial fat and on the surface of the endothelium. The tissue of the aortic and mitral valves and their base appeared swollen and poor in cells.

The rats fed 150 mg. of the vitamin E concentrate once weekly, but no cholesterol and bile salt (group 3, E-high, table), showed overdevelopment of collagenous tissue at the base of the valve and in the medial coat of the aorta. In the intima of the ascending aorta and the dorsal arc there were sclerotic foci, cellular in female, larger and hyalin-like in male, animals. Close by in the media there were often vasa vasorum. The sclerotic foci and the acacia of the surrounding medium were imbued with chloroform-soluble, highly birefringent material, presumably cholesterol ester. With time, this substance was seen to cover an increasingly large area and fade out. The endothelial cells covering the marginal part of the foci and those in other spots were loaded with fat and phospholipids or they were replaced by foam cells. But over the centers of the foci the endothelium had degenerated and only free fat droplets remained.

The rats of group 4 (E-high, C + B), which were fed the same amount of vitamin E concentrate but, in addition, were given cholesterol and bile salt, showed only some vasa vasorum and slight fat infiltration of the endothelium, not exceeding that found in controls. The transparency of the aortic wall was not perceptibly increased after chloroform extraction, indicating a singularly low fat content of the media.

COMMENT

Neither vitamin E deficiency nor an excessive dose of the vitamin E concentrate modified cholesterol levels in blood, liver or adrenal glands of rats significantly, in agreement with observations by Monnier and co-workers[1] and Dam.[2] Our findings disagree, however, with results obtained by Morgulis and Spencer,[3] who observed a rise in blood cholesterol of rabbits in muscular dystrophy. Whether the discrepancy is due to the difference in species or to a difference in experimental conditions is not known.

In contrast to the chemical findings, the morphologic examination of the aorta of the vitamin E–deficient animals showed a tendency toward fatty infiltration not accompanied by deposition of cholesterol. This imbibition of fat was correlated neither with the cholesterol levels of blood or liver nor with the cholesterol and bile salt contents of the diet. The phenomenon may be related, however, to the increased permeability of capillaries observed by Dam and Glavind[15] in vitamin E–deficient animals; whether it is also related to Holman's recent finding[16] that vitamin E prevented experimental arteritis in dogs is not known.

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14. Leffkowitz, M., and Rosenberg, D.: Frankfurt. Ztschr. f. Path. **34**:174, 1926.

15. Dam, H., and Glavind, J.: Nature, London **143**:810, 1939.

16. Holman, R. L.: Proc. Soc. Exper. Biol. & Med. **66**:307, 1947.

An excess of the vitamin E concentrate proved more harmful than the deficiency. In the group fed an excess of the vitamin E concentrate but no cholesterol plus bile salt, the aorta showed in 6 of 7 animals sclerotic patches which contained cholesterol deposits. These lesions were relatively small and not comparable to those produced, for example, by cholesterol feeding of rabbits or chickens. But it should be stressed that they developed without added dietary cholesterol and that in the males they exceeded in severity the slight pathologic changes of the rat aorta so far produced experimentally by the authors.

No other toxic effect was observed as a result of the high level of the vitamin E concentrate except the mentioned genital symptoms. The animals appeared in a good general condition, and their growth curves resembled those of normal animals.

It has to be emphasized that the vegetable oil concentrate contained 66 per cent of material other than vitamin E and that it is not known at present whether the tocopherols or other components of the concentrate were responsible for the toxic action. As certain oil fractions,[17] in particular, some of the unsaturated fatty acids,[18] are known to cause arterial lesions, and since Holman reported recently on the beneficial effects of vitamin E in the case of arterial lesions produced by renal damage and cod liver oil,[19] it is imperative to repeat the experiment described here with pure tocopherol before further conclusions are drawn.

If it was unexpected to find aortic lesions in the group receiving the high dose of the vitamin E concentrate but no extra cholesterol in the diet, it was even more surprising to observe that a high intake of cholesterol and bile salt completely prevented such damage. The last experimental group, receiving the same, apparently toxic level of the vitamin E concentrate but, in addition, 1 per cent of cholesterol and 0.5 per cent of bile salt with the diet, showed only mild fatty infiltration but neither sclerosis nor cholesterol deposition in the aorta. It is not known whether the cholesterol or the bile salt was responsible for the protective action. It is interesting, in connection with this observation, that Holman reported recently that arterial lesions produced by renal damage and cod liver oil could be prevened or retarded by dietary cholesterol.[19]

SUMMARY

The influence of vitamin E (alpha tocopherol) on cholesterol metabolism and the development of arteriosclerosis was investigated in the rat. Neither vitamin E deficiency nor an excess of a vitamin E concentrate modified blood or tissue cholesterol levels. Avitaminosis E caused fatty infiltration of the wall of the aorta, regardless of the cholesterol content of blood or diet. High doses of a vitamin E concentrate produced localized sclerosis and deposition of cholesterol in the aorta. These changes were prevented by high dietary cholesterol and bile salt.

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Excessive Dietary Vitamin E: Its Alleviation of Hypervitaminosis A and Lack of Toxicity

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HYPERVITAMINOSIS A has been reported in several species particularly in domestic pets (Seawright *et al.*, 1965) which were fed large amounts of liver, and in humans (Persson *et al.*, 1965) due to therapeutic overdoses of vitamin A. High levels of this vitamin were also shown to have a detrimental effect on calcification in cows (Manston, 1966). On the other hand, moderate levels of vitamin A have been used experimentally in combination with massive doses of vitamin D to treat milk fever in lactating cows (Payne and Manston, 1967) without toxic effects of either vitamin. This antagonistic action of vitamins A and D on the toxicity of each other has been studied in chicks by Taylor *et al.* (1968). The present report indicates that the toxicity due to excessive vitamin A can be counteracted by large doses of vitamin E.

Preliminary studies indicated that a high level of dietary vitamin E ameliorated the growth depression caused by excess dietary vitamin A and the following experiment was conducted to confirm the relationship between the two vitamins at high levels. A practical corn-soybean type of ration (Motzok *et al.*, 1967) containing adequate amounts of vitamins A and E was used as the control diet. Experimental diets vitamin A (gelatinized beadlet form) was added at a level of 3,250,000 I.U./kg. of diet and vitamin E as pure dl-alpha tocopheryl acetate was dissolved in the soybean oil portion of the diet to give a final level of 10,000 I.U./kg. (1.0% of the diet). These levels of vitamins A and E are approximately a thousand times the normal optimum amounts. The dietary treatments were as shown in Table 1. Each diet was fed to 2 pens of 9 Vantress × White Plymouth Rock cockerels from 1 day of age. The birds were reared in electrically heated wire-floored battery brooders and the diets were allotted at random.

TABLE 1.—*Effect of excesses of vitamins A and E on livability of chicks*

Dietary treatments	Number of chicks surviving	
	18 days	32 days
1. Control diet.	18	18
2. Control+vitamin E	17	17
3. Control+vitamins A and E	18	18
4. Control+vitamin A	16	4
5. Diet 4 changed to diet 1 at 18 days	18	10
6. Diet 4 changed to diet 2 at 18 days	18	14

The data in Table 1 show that the mortality at 32 days was high on the vitamin A diet. Simultaneous feeding of large amounts

of vitamin E with vitamin A (treatment 3) completely prevented this effect. Chicks fed excess vitamin E alone had normal livability.

High dietary vitamin A also markedly depressed the growth of chicks (Fig. 1) and this was largely overcome by the inclusion of excess vitamin E throughout the feeding period. However, recovery from vitamin A-induced growth depression after removal of the vitamin A at the 18th day was not facilitated by excess vitamin E (treatment 6 *vs.* 5). In contrast to the toxicity of vitamin A, excessive dietary vitamin E alone had no detrimental effect on the growth of the chicks.

The livers of birds fed high vitamin A alone were small and clay-coloured. Those of the high vitamin E animals were larger and darker red than the controls. The control and vitamin A + E livers appeared similar (the gross- and histopathology of hypervitaminosis A in chicks will be reported elsewhere).

Cox *et al.* (1957) found that some antioxidants potentiated the toxic effect of excessive vitamin A in rats, however tocopherols did not. The present finding that very high levels of vitamin E actually reduced the severity of hypervitaminosis A may indicate that the physiological function of vitamin E is other than merely a tissue antioxidant as suggested by some workers (see Tappel, 1962). On the other hand, if the protective action of vitamin E is due to its antioxidant properties, it may indicate that the pathology due to excessive vitamin A is an oxidative destruction of tissues.

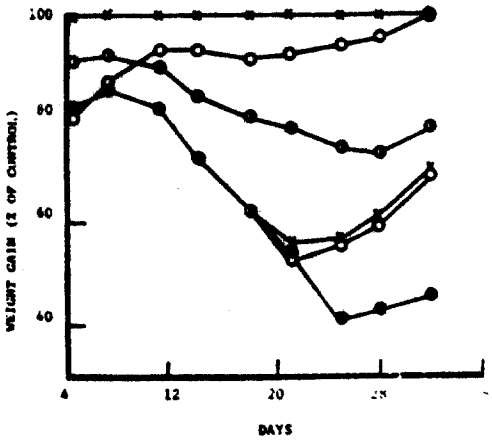

FIG. 1. Effect of excesses of vitamins A and E on the growth of chicks. Control diet, ×, vitamin E, ●; vitamins A and E, ◑; vitamin A, ● (means of 6 groups of chicks for 18 days and 2 groups for the remaining period).

Antioxidants have been found to increase the mean life span of mice when added to the diet (Harman, 1969) and it is suggested that such additions to human diets may eventually increase the useful lifespan of man. The present finding that vitamin E, a physiological antioxidant, is virtually non-toxic in massive doses may be significant in the light of the above suggestion.

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Effect of Supplementing the Diet of Man with Tocopherol on the Tocopherol Levels of Adipose Tissue and Plasma

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Several investigators have shown that the tocopherol requirements in animals and birds are increased when their diets contain large amounts of unsaturated fats.[1] The same phenomenon might be expected to occur in man in which case greater attention than heretofore should be accorded to tocopherol intakes, particularly if it should prove reasonable to continue to advocate the ingestion of polyunsaturated fats.

These studies were undertaken in order to determine the effect of supplementing ordinary hospital diets with liberal amounts of tocopherol on the levels of tocopherol in adipose tissue and plasma.

METHOD

Nineteen hospitalized male patients, aged thirty to eighty-one, were maintained on mixed diets of varying composition. Table 1 includes the medical diagnoses. Plasma and subcutaneous adipose tissue samples were obtained before and two weeks after each patient took 1 gm. of tocopherol daily. Eprolin℠ (Eli Lilly and Co.) is available as capsules each containing 60 mg. *d*-alpha-tocopherol plus varying amount of beta, gamma and delta tocopherols to a total of 100 mg. Patients received 5 capsules, twice daily.

Plasma samples were analyzed according to the method used by Leitner et al.,[2] modified as follows: instead of correcting for carotenoids, interfering substances were removed by column chromatography as described by the Analytical Methods Committee.[3] The column was packed with Floridin Earth XS especially prepared for vitamin E analysis (British Drug Houses, Ltd.). Interfering sterols, carotenoids and vitamin A were absorbed. Tocopherol was estimated by the ferric chloride-dipyridyl reaction of Emmerie-Engle. Optical density was measured at 520 mμ in a Bausch-Lomb spectrophotometer. The standard curve was prepared with alpha-tocopherol.

Adipose tissue obtained as described heretofore[4] was weighed (sample weights, 300 to 1,000 mg.) and extracted with chloroform-methanol. The residue was weighed and saponified. The unsaponifiable fraction was recovered, dissolved in methanol for removal of steroids, evaporated, redissolved in benzene and subjected to column chromatography as described. Tocopherols were recorded as micrograms per gram of adipose tissue. Recoveries of added tocopherol were in the range 90 to 105 per cent.

RESULTS AND DISCUSSIONS

The results are presented in Table 1. Before supplementation the plasma tocopherol levels ranged from 1 to 13 μg. per ml., with an average of 4 μg. per ml. These are lower than previously reported normal values, stated to be about 10 μg. per ml.,[2] and also lower than those of a control series of thirty hospital patients reported by us (8.5 μg. per ml.).[4] However Herting and Drury[5] have found an average of 4.3 μg. per ml. in the plasmas of seventy-four normal human adults. They interpret their low values as reflecting more accurate estimates

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TABLE I
Plasma and Adipose Tocopherol Content Before and After Tocopherol Supplementation

Patient No.	Age (yr.)	Plasma (μg./ml.) Start	Plasma (μg./ml.) End*	Adipose Tissue (μg./mg.) Start	Adipose Tissue (μg./mg.) End*	Brief Diagnosis
1	30	3	16	18	129	Pancreatitis
2	34	2	19	57	234	Leukemia
3	40	2	5	29	43	Alcoholism
4	42	9	20	45	105	Ulcer; alcoholism
5	44	5	10	31	68	Alcoholism
6	45	1	5	33	53	Alcoholism
7	48	1	3	14	46	Diabetes
8	52	2	5	35	25	Pneumonia
9	53	1	5	28	78	Hypothyroidism
10	56	9	15	97	19	Hyperthyroidism
11	59	4	10	14	27	Alcoholism
12	62	3	13	122	291	Ulcer
13	68	13	33	201	336	Heart disease
14	68	3	9	30	158	Pneumonia
15	71	1	18	14	132	Dermatitis
16	73	3	10	48	83	Heart disease
17	77	4	7	62	183	Ulcer
18	78	3	6	43	175	Heart disease
19	81	3	7	68	88	Heart disease
Averages	56.9	3.7	11.3	52	120	...

* After two weeks of daily supplementation with 1 gm. of tocopherol.

of the actual tocopherol content as compared with those obtained by methods used by previous investigators.

Dju et al.[6] previously suggested that disease may cause a depression in tissue tocopherol levels, but our data are insufficient to establish this as the cause for our low values. Supplementation increased the plasma tocopherol levels to an average of 11 μg. per ml. (range 3 to 33 μg. per ml.), but in eight patients the levels were still below the normal range.

Before supplementation adipose tissue tocopherol levels ranged from 14 to 201 μg. per gm., with an average of 52 μg. per gm. (compared to an average of 144 μg. per gm. in the previous series[4]). After supplementation the levels ranged from 27 to 336 μg. per gm. with an average of 120 μg. per gm. The large drop and low final value noted in one patient (No. 10) are not explained. In the other patients no influence of age on the original tocopherol level or on the magnitude of the increase was apparent. Assuming that adipose tissue accounts roughly for 10 per cent of the body mass, an average of approximately 0.5 gm. of tocopherol accumulated in the adipose tissue after the administration of 14 gm. over a period of two weeks. Further work to determine the permanence of such increased tocopherol levels is in progress.

SUMMARY

Nineteen hospital patients were given 1 gm. of mixed tocopherols orally, every day for two weeks. Tocopherol levels in plasma and adipose tissue were determined at the start and immediately after the last supplement was taken. Average plasma tocopherol levels increased from 3.7 to 11.3 μg. per ml. and adipose tocopherol levels increased from 52 to 119 μg. per gm.

ACKNOWLEDGMENT

We are indebted to Eli Lilly and Company for the vitamin E preparation used in this study.

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Tocopherol Storage and Depletion in Adipose Tissue and Plasma of Normal and Diabetic Human Subjects[1,2]

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IT WAS SHOWN IN A PREVIOUS STUDY of hospitalized patients that the tocopherol levels of adipose tissue and plasma were markedly increased when the ordinary hospital diet was supplemented with liberal amounts of tocopherol (1). It appeared of interest to determine whether similar increases could be induced in healthy subjects and also how rapidly the tocopherol levels would decrease after its discontinuance. In this paper we report such a study involving 18 apparently healthy male subjects between the ages of 30 and 40. Results with 20 obese diabetics are also presented.

METHODS

The 18 normal male subjects were volunteers from the Vacaville Medical Facility, a California correctional institution. They consumed an institutional diet in which the fat was derived principally from animal fat and hydrogenated shortenings.

At the start of the study samples of subcutaneous adipose tissue (0.5–1.0 g) and of fasting blood were taken from each subject. All subjects were then given 1 g of tocopherol (Eprolin, Eli Lilly & Co.) daily, one 500-mg capsule each morning and one each evening for 14 days. Samples of adipose tissue and plasma were obtained at the end of the period of supplementation and again 2 weeks after supplementation had ended.

The 17 female and 3 male obese nonpro ketotic diabetics were clinic patients of a large county hospital who lived at home. Each had received diet instructions, which varied according to the degree of hyperglycemia and obesity, but dietary control was less than optimal. Fourteen received some medication in addition to the tocopherol supplement.

Methods of extraction and analysis were the same as those described previously (1). The extracted lipid from the tissue was saponified for 5 min with aqueous potassium hydroxide and ethanol containing pyrogallol (see also Diplock et al. (2)). Sterols were precipitated from both plasma and tissue extracts with methanol. Other interfering substances were removed by column chromatography. Quantitative determinations of tocopherol were made by the Emmerie-Engel reaction.

RESULTS AND DISCUSSION

Normal Males

The results with the group of 18 normal males are shown in Table 1. The tocopherol levels of the adipose tissue samples before supplementation ranged from 11

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[2] These studies were supported in part by National Institutes of Health Grant HE-00955.

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TABLE 1

Tocopherol Content of Adipose Tissue and of Plasma of Normal Males before and after Tocopherol Supplementation

Subject	Tissue μg/g[a]			Plasma μg/ml[a]		
	A	B	C	A	B	C
1	78	127	81	3	7	5
2	65	81	77	3	17	11
3	32	70	59	5	14	12
4	38	145	72	1	13	7
5	34	81	58	4	14	8
6	17	63	29	5	9	4
7	49	83	58	1	11	8
8	47	53	46	3	10	5
9	49	65	47	4	9	6
10	43	86	57	3	7	4
11	31	99	87	4	8	6
12	17	87	74	3	7	6
13	45	100	57	3	15	10
14	14	48	32	2	5	5
15	14	49	33	2	8	6
16	24	94	44	9	14	4
17	30	42	37	9	18	12
18	59	61	44	9	15	7
Average[b]	38.1 ± 17.6	79.7 ± 26.6	55.1 ± 17.0	4.1 ± 2.5	11.2 ± 3.9	7.0 ± 2.6

[a] A, before supplementation; B, at end of 2-week supplementation with 1 g tocopherol daily; C, 2 weeks following supplementation.

[b] Averages ± standard deviation.

μg/g to 78 μg/g, average, 39 μg/g. The levels at the end of the period of supplementation ranged from 42 to 145 μg/g, average, 80 μg/g, approximately double the initial value. In the previous study with hospitalized patients the original value for adipose tissue was 52 μg/g, and after 2 weeks supplementation, 120 μg/g. In the present study, 2 weeks after the cessation of supplementation the adipose tissue tocopherol range was 15 to 87 μg/g; average, 54 μg/g.

The plasma tocopherol levels for the same three sampling periods are also shown in Table 1. The average at the first analysis was 4 μg/ml. Herting and Drury (3) recently reported plasma values of 3.58 ± 0.21 μg/ml for one group of subjects (35 male, 2 female) and 5.07 ± 0.32 μg/ml for another (25 male, 12 female). Dayton et al. (4) found plasma values that ranged from 1.57 μg/ml to 3.05 μg/ml, average 2.31 μg/ml, for seven elderly male subjects. Previous reports (5) of averages of about 10 μg/ml for normal subjects possibly reflect errors in the methodology used in the earlier work. Binder et al. (6) suggested that serum levels of tocopherol below 2.25 μg/ml were indicative of tocopherol deficiency in man.

In the present study the average values in plasma after supplementation were 11 μg/ml, approximately a three-fold increase, similar to the increase from 3.7 to 11.3 μg/ml found in the study with hospitalized patients (1). Two weeks after supplementation had ended the average plasma-tocopherol level was at 7 μg/ml.

TABLE II

Tocopherol Levels of Adipose Tissue and of Plasma of Diabetic Patients before and after Tocopherol Supplementation

Subject	Degree of Obesity[b]	Age and Sex	Tissue μg/g[a]			Plasma μg/ml[a]		
			A	B	C	A	B	C
19	1	56 F	30	89	36	8	16	6
20	2	76 M	17	105	40	5	12	8
21	2	63 F	27	36	21	8	13	5
22	2	51 F	17	174	116	12	24	14
23	2	64 F	35	57	20	4	12	2
24	2	55 F	15	67	21	9	14	9
25	2	72 F	56	187	114	11	31	20
26	2	50 F	46	94	91	8	22	9
27[c]	2	69 F	48	69	70	6	16	11
28	2	72 M	10	88	21	8	22	13
29	2	67 F	11	57	38	19	22	13
Average		63	28 ± 15	93 ± 51	55 ± 36	9 ± 4	19 ± 6	10 ± 5
30	3	53 F	32	73	23	4	8	7
31[c]	3	32 M	43	165	44	17	14	7
32	3	56 F	59	67	41	8	22	8
33	3	39 F	35	91	47	8	18	8
34	3	55 F	17	55	29	5	16	7
35[c]	3	49 F	27	130	96	6	24	11
36	3	59 F	35	62	37	14	21	12
Average		49	35 ± 10	92 ± 38	45 ± 22	9 ± 4	18 ± 5	9 ± 2
37	4	53 F	6	49	17	4	14	11
38[d]	4	42 F	47	93	51	14	22	12
Average		48	26 ± 20	71 ± 22	34 ± 17	9 ± 5	18 ± 4	12 ± 1
Total average		57	31 ± 15	90 ± 45	49 ± 31	9 ± 4	18 ± 5	10 ± 4

[a] See footnote, Table I.

[b] Arbitrarily assigned from 1, moderate, to 4, very obese; based on body weight and subjective appraisal by one individual.

[c] *Subject 27* on Dimelor (acetohexamide), *31*, an alcoholic on unknown medication, and *35*, on insulin, are not included in Table III.

[d] Pregnant.

Diabetic Patients

The wide variations in tocopherol levels in the diabetic patients (Table II) may have reflected differences in the state of health of the subjects, such as nutritional status and amount of control of diabetes. The high value of 14 μg in the plasma of one pregnant patient in the present study is in accord with the suggestion that tocopherol level may be elevated in such cases (Darby et al. (7)). Vanzetti and coworkers (cited in (5)) found that the mean plasma-tocopherol level in diabetics was higher than in healthy subjects.

The range of the adipose tissue-tocopherol levels of the diabetic patients in the present study was 6 to 59 μg/g; average, 31 μg/g; compared to 39 μg/g in the non-

TABLE III

Comparison of Average Tocopherol Levels in Groups of Diabetic Patients

Variable Factor	Number of Patients	Tissue μg/g[a]			Plasma μg/ml[a]		
		A	B	C	A	B	C
No medication[b]	6	24	48	33	9	17	9
Tolbutamide[c]	8	29	96	54	10	19	10
Chlorpropamide[d]	3	33	69	47	6	17	6
Obese 1 and 2	11	28	93	55	9	19	10
Obese 3	7	35	92	45	9	18	9
Normal male (Table 1)	18	39	80	54	4	11	7
Diabetics (Table II)	20	31	90	49	9	18	10

[a] See footnote, Table 1.
[b] *Subjects 19, 21, 24, 28, 33, 38.*
[c] Tolbutamide is Orinase (Upjohn), *subjects 20, 22, 25, 29, 30, 32, 36, 37.*
[d] Chlorpropamide is Diabinese (Pfizer), *subjects 23, 26, 34.*

mal male subjects. The adipose tissue-tocopherol levels before, during, or after tocopherol supplementation did not appear to correlate with the degree of obesity (Table II). The tocopherol range in adipose tissue after supplementation was 36 to 187 μg/g; average, 92 μg/g. Two weeks after supplementation had ceased the range was 21 to 116 μg/g; average, 45 μg/g.

Table III shows results for subjects arranged in groups according to medication. The average adipose tissue-tocopherol values at the first sampling are similar for all groups, but at the second sampling, after supplementation, the group using an oral hypoglycemic agent (tolbutamide) had an average tocopherol level twice that of the group without medication. The respective values were 96 μg/g and 48 μg/g. After supplementation had been discontinued for 2 weeks, the average tocopherol values were 54 μg/g for the subjects taking oral hypoglycemic agents and 33 μg/g for those without medication. Thus within this very limited series, the results suggest that hypoglycemic agents may have some influence on absorption and retention of tocopherol.

The average plasma-tocopherol level of the diabetics before supplementation was 9 μg/ml, compared with 4 μg/ml found in the normal males. After supplementation the average plasma-tocopherol value for the diabetic group was 18 μg/ml. The average plasma-tocopherol level 2 weeks after supplementation was 10 μg/ml, nearly equal to the 9 μg/ml found at the beginning of the study. In contrast with the group of normal males, the decline after supplementation ceased was from 11 μg/ml to 7 μg/ml, still higher than the original plasma level of 4 μg/ml.

In the late 1940's several authors claimed that clinical improvement could be achieved by supplementing the diets of diabetics with tocopherol but a careful study of 35 patients by Bensley et al. (8) indicated that such treatment was not demonstrably beneficial, except to double the plasma-tocopherol levels. In the present series also no signs of improvement were noted.

The fate of tocopherol in various tissues of the rat and chicken has been studied with ^{14}C tocopherol by Krishnamurthy and Bieri (9). Twenty-four hours after oral administration more than half of the total α-tocopherol dose was recoverable from all tissues. Different tissues varied greatly in their rate of uptake and depletion. After 21 days the total tocopherol in all tissues was approximately 25% of that accounted for at the end of 24 hr.

SUMMARY

The results of dietary supplementation with tocopherol in the human on tocopherol levels in adipose tissue and plasma were observed with 18 normal males (30–40 years) and also in 20 obese diabetics.

In the subjects in normal health, 2 weeks of supplementation with 1.0 g of tocopherol/day for each subject increased the tocopherol levels of adipose tissue from 38 to 80 μg/g and of plasma from 4–to–11 μg/ml. The levels declined rapidly when supplementation ceased.

With the group of diabetics the tocopherol levels of adipose tissue and plasma were similarly substantially increased by oral supplementation. In adipose tissue the relative increase in the diabetics was apparently greater than in the normal subjects and the rate of depletion seemed more rapid. Medication with oral hypoglycemic agents appeared to be associated with higher levels of tocopherol during and following supplementation. The relative increase of tocopherol in plasma of the diabetic subjects was found to be smaller and the rate of depletion after the end of supplementation was more rapid than with the normals. However, the number of patients studied was too small and the number of variables too large to permit more than suggestions for further investigation.

We are grateful to Dr. William C. Keating, Director of the California Medical Facility, Vacaville, at the time the study of the normal men was initiated; to the staff of the Facility and the volunteers for their very helpful cooperation; and to Eli Lilly & Co. for financial support and for the tocopherol preparation used.

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The distribution and metabolism of α-tocopherol in the rat

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Biochemistry Department, University of Liverpool

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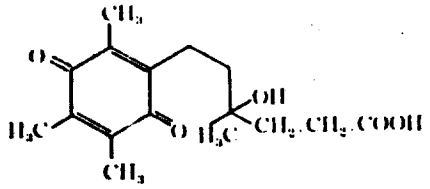
1. A comparison of the distribution of radioactivity in rat tissue lipids after oral administration of [5-*Me*-^{14}C]-α-tocopherol showed that spleen and adrenal lipids have a relatively high uptake of radioactivity. 2. Absorbed α-tocopherol and its metabolites appeared not to re-enter the lumen of the gut in significant amount through the bile or by the secretion from the stomach mucosal cells within the 24 h experimental period. 3. After ingestion of [5-*Me*-^{14}C]-α-tocopherol by rats, analyses of their liver and spleen lipids showed that most of the radioactivity in the lipids was due to the presence of unchanged α-tocopherol. Radioactive tocopherolquinone was found in both spleen and liver lipids; in the liver lipids there was some evidence that a second oxidation product of α-tocopherol, compound O, had been formed.

There is conflicting evidence about the existence in the animal body of α-tocopherol metabolites. Simon, Gross & Milhorat (1956) reported finding a metabolite of α-tocopherol, later called tocopheronic acid, and its lactone in the urine of rabbits given [5-*Me*-^{14}C]-D-α-tocopherol succinate by intravenous or subcutaneous injection. Simon, Eisengart, Sundheim & Milhorat (1956) isolated these compounds from the urine of human subjects given 3–5 g D-α-tocopherol daily and identified them as 2-(3-hydroxy-3-methyl-5-carboxyl-pentyl)-3,5,6-trimethyl-1,4-benzoquinone (I) and



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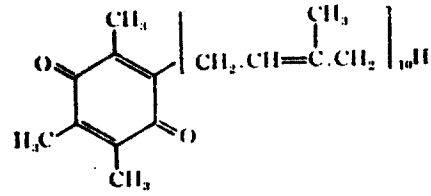

II

its γ-lactone. It was at one time suggested that tocopheronic acid was the active form of vitamin E or closely allied to it (Green, Edwin, Diplock & Bunyan, 1961; Green, Diplock, Bunyan, Edwin & McHale, 1961) but so far it has only been found as a urinary metabolite.

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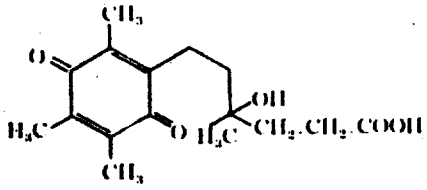

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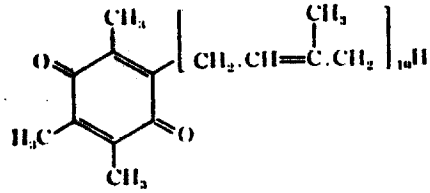

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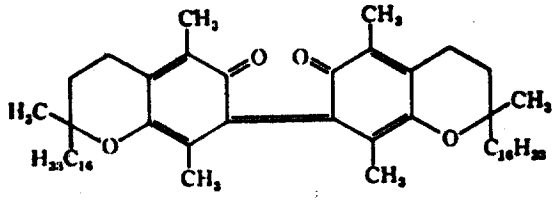
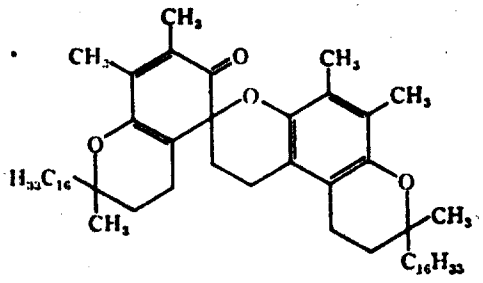
Martius & Fürer (1963) identified a metabolite of α-tocopherol, found in the tissues of rat, chick and earthworm, as 2,5,6-trimethyl-3-(farnesylfarnesylgeranylgeranyl)-1,4-benzoquinone (II) referred to as $E_{\alpha(50)}$ by analogy with the vitamins K and suggested that it was the metabolite of α-tocopherol responsible for specific vitamin E activity as apart from non-specific antioxidant activity. It is perhaps significant that tocopheronic acid is an intermediate in the proposed scheme for the interconversion of α-tocopherol to $E_{\alpha(50)}$.

Two oxidation products of α-tocopherol have been reported in animal tissues. The first of these, tocopherolquinone, has been reported by several authors. Morton & Phillips (1959) found tocopherolquinone in rat liver and trace amounts in rat kidney and rat heart. Draper, Csallany & Shah (1962) and Plack & Bieri (1964) found tocopherolquinone in the livers of rats, previously deficient in vitamin E, given [5-*Me*-^{14}C]-α-tocopherol. Weber & Wiss (1963) found α-tocopherolquinone in the depot fats of rats, previously deficient in vitamin E, but were unable to find any metabolites in the liver, or in the brain.



III

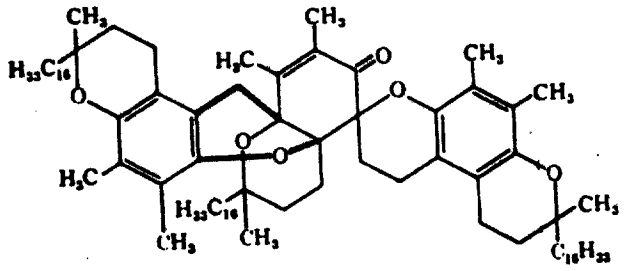


IV

Alaupović, Johnson, Crider, Bhagavan & Johnson (1961) found three labelled compounds in the livers of rats and pigs given [^{14}C]-α-tocopherol by intraperitoneal injection. Also Draper *et al.* (1962) isolated one of these metabolites, compound O, from the livers of rats previously made deficient in vitamin E and given [5-*Me*-^{14}C]-α-tocopherol, and showed it to be identical with a product prepared in vitro by oxidizing α-tocopherol with potassium ferricyanide. Csallany & Draper (1963) identified this as a dimeric oxidation product of α-tocopherol to which they assigned the structure (III). But Nelan & Robeson (1962) suggested that the main product of in vitro oxidation of α-tocopherol with alkaline ferricyanide was a dimeric keto ether (IV); this structure was proved by Schudel, Mayer, Metzger, Rüegg and Isler (1963). Skinner & Alaupović (1963) investigated the products obtained when α-toco-

pherol was oxidized with alkaline ferricyanide and found that 65–75 % of the total product was dimeric, as structure IV, but 2–3 % was in the form of a trimeric oxidation product (V). Further evidence for this structure was provided by Skinner & Parkhurst (1964) working with an analogue of α-tocopherol. Skinner & Alaupović found that the ultraviolet absorption spectrum, infrared absorption spectrum and paper chromatogram of the trimeric product, rather than the dimeric product, resembled that of compound O.

The aim of the study now presented was to trace the route of α-tocopherol in rats free from any nutritional deficiency so that normal patterns of distribution and metabolism of the vitamin could be investigated.



V

EXPERIMENTAL

Norwegian hooded rats from the stock colony kept in this department were used throughout. They were maintained on food cubes (No. 4 diet; British Extracting Co. Ltd, Bromborough Port, Cheshire). Diet and water were given *ad lib.*

Isotopically labelled α-tocopherol was prepared from [5-*Me*-^{14}C]-α-tocopherol succinate (Distillation Products Industries, Rochester, NY) by reduction with lithium aluminium hydride by the method of Duggan (1959), and its purity was established by chromatography on zinc carbonate-impregnated paper.

Twelve male rats, 4–6 months old, were each given by stomach tube 0·5 ml of a solution of [5-*Me*-^{14}C]-α-tocopherol in arachis oil (0·36 μc ml). Groups of three rats were killed 3, 6, 12 or 24 h after dosing. When the interval between dosing and killing exceeded 12 h the animals were fitted with aluminium tail cups to prevent coprophagy. Before each animal was killed and while it was under anaesthesia its bile was collected for 1 h by means of a cannula inserted into the common bile duct.

Organs from the three rats killed at each time interval were dissected out and pooled. The collected tissues were the liver, kidneys, spleen, adrenals, testes, pelt, stomach, intestine (from the duodenum to the colon) and the caecum. The rest of the carcasses were pooled, minced finely in a hand mincer and mixed thoroughly; a large sample was taken for extraction.

The tissues were homogenized in 20 ml chloroform–methanol (2:1, v/v) per g tissue and lipids were extracted into chloroform by the technique of Albrink (1959–60).

The radioactivity of a small measured portion of each chloroform extract was measured in a Packard Tri-Carb liquid scintillation counter. A minimum of 5000 counts/min above the background was recorded for each sample.

Livers and spleens from ten male rats killed 24 h after being given [5-*Me*-^{14}C]-α-tocopherol orally were homogenized in isotonic sucrose at 0°, and cell particles were separated by centrifugation by the method of Schneider (1948). Lipids were extracted from each fraction (Folch, Lees & Stanley, 1957), and the radioactivity of each fraction was determined as before.

To identify the radioactive material in the liver and spleen, forty-six female rats were given 0·36 μc [5-*Me*-^{14}C]-α-tocopherol by stomach tube and were killed 24 h later. The livers and spleens were removed, and lipids were extracted into chloroform by the technique of Albrink (1959–60). These lipids were then separated by chromatography on acid-washed alumina (Brockman grade III) with mixtures of diethyl ether in light petroleum (b.p. 40–60°) of increasing polarity to elute the lipids. Radioactive fractions were subjected to further purification on 10 g columns of Decalso F (Permutit Co.) and finally separated by thin-layer chromatography and paper chromatography. Lipid was separated on thin layers of silica gel G (Merck) developed with 15% (v/v) ethyl acetate in benzene. The paper-chromatographic separation was a modification of the method recommended for the analysis of tocopherols by the Society for Analytical Chemistry: Analytical Methods Committee (1959). Lipid dissolved in *cyclo*-hexane was spotted on 20 cm² zinc carbonate-impregnated paper (Whatman no. 1) and developed in the first dimension by 1% (v/v) acetone in light petroleum. After impregnating with liquid paraffin, the chromatogram was developed in the second dimension with 95% (v/v) aqueous ethanol.

RESULTS

Distribution of radioactivity

The distribution of radioactivity in rat tissues after oral administration of [5-*Me*-^{14}C]-α-tocopherol, expressed as counts/min g tissue, counts min g extracted lipid and as a percentage of the dose, is shown in Table 1.

Most of the radioactivity found 3 h after the administration of the dose was in the stomach and intestine, presumably in the contents. These levels fell rapidly in the next 3 h, whereas the radioactivity in the caecum increased from 2·2% of the dose to 20·3%. Presumably between 6 and 12 h much of the radioactivity was voided with the faeces. After 24 h only about 20% of the administered radioactivity was recovered from the body.

No significant radioactivity was detected in bile samples collected in the hour before slaughter (Table 2).

When the distribution of radioactivity in the tissues is expressed in the three ways listed above, it is seen that, although, the adrenal and the spleen, for example, take up most radioactivity relative to their weight and lipid content, because of their relatively low weights the fraction of the dose taken up by them is small. As might be expected, the liver takes up the largest percentage of the dose. Table 3 lists organs in descending

order of uptake calculated in these three ways after 24 h; stomach, intestine and caecum are not included.

Table 4 shows the intracellular distribution of the radioactivity found in rat liver and spleen. There was a clear difference in distribution. In the spleen 59·3 % of the recovered ^{14}C was associated with the mitochondrial fraction, 18·8 % with the microsomal fraction. In the liver, the microsomal fraction contained a bigger proportion of ^{14}C found, 43·7 % compared to 12·2 % in the mitochondrial fraction.

Table 1. *Distribution of radioactivity in rat tissues after oral administration of* 0·18 *μc* [5-Me-^{14}C]-*α-tocopherol* 3, 6, 12 *and* 24 *h after dosing*

	Radioactivity											
	Counts/min g tissue				Counts/min g lipid				% of dose			
Tissue	3 h	6 h	12 h	24 h	3 h	6 h	12 h	24 h	3 h	6 h	12 h	24 h
Liver	260	885	482	810	6850	22200	16300	9580	1·96	4·20	2·80	3·74
Kidney	37	448	105	190	69	9170	4430	5120	0·05	0·39	0·11	0·17
'Stomach'*	3100	2040	305	126	60000	36200	10900	2290	13·07	3·30	1·05	0·29
'Intestine'*	2920	963	467	346	17080	7060	2340	1600	16·24	3·75	2·71	1·67
'Caecum'*	405	5170	1263	551	7720	95500	14450	6390	2·17	20·28	5·43	2·00
Testis	7	134	68	91	299	3720	2520	2830	0·00	0·15	0·09	0·10
Spleen	387	986	1110	1271	13640	46200	27500	28700	0·09	0·25	0·25	0·19
Adrenal	543	1510	5380	1675	4550	6970	38600	22280	0·01	0·03	0·11	0·03
Pelt	110	85	103	166	1875	685	995	1920	2·72	1·58	2·50	3·52
Rest of carcass	392	93	216	148	3820	1569	1935	1225	30·40	7·48	15·55	8·22
								Total recovery	66·71	41·41	30·60	19·93

* Includes contents at time of killing.

Table 2. *Radioactivity found in individual samples of rat bile after oral administration of* 0·18 *μc* [5-Me-^{14}C]-*α-tocopherol*

	Time after dose			
	2–3 h	5–6 h	11–12 h	23–24 h
Wt of bile collected during 1 h (g)	0·4238	0·5976	0·2177	0·6098
		0·5697	0·5160	0·3765
Activity: (counts/min above background)	28	64	22	11
	—	101	12	25
% dose	0·012	0·027	0·009	0·005
	—	0·043	0·005	0·011

Table 3. *Rat tissue listed in order of relative radioactivity content* 24 *h after the oral administration of* [5-Me-^{14}C]-*α-tocopherol*

Counts/min g tissue		Counts/min g lipid		% of dose	
Adrenal	1675	Spleen	28700	Liver	3·74
Spleen	1271	Adrenal	22280	Spleen	0·19
Liver	810	Liver	9580	Kidney	0·17
Kidney	190	Kidney	5120	Testis	0·10
Testis	91	Testis	2830	Adrenal	0·03

Table 4. *Intracellular distribution of radioactivity in rat liver and spleen tissue 24 h after oral administration of* [5-Me-^{14}C]-α-*tocopherol*

Tissue	Radioactivity		Tissue	Radioactivity	
	Counts/min	% total		Counts min	% total
Spleen:			Liver:		
Nuclei	1846	9·6	Nuclei	330	13·5
Mitochondria	11325	59·3	Mitochondria	300	12·2
Microsomes	3601	18·8	Microsomes	1070	43·7
Supernatant fraction	2334	12·2	Supernatant fraction	755	30·6

Liver analysis

Eight livers were obtained from rats that had been given [5-*Me*-^{14}C]-α-tocopherol 24 h before death, and the chloroform-soluble liver lipids were extracted. Of the radioactivity found in the chloroform extract 97% was soluble in acetone. The acetone-soluble lipid was chromatographed on acid-washed alumina and eluted with mixtures of diethyl ether in light petroleum of increasing polarity. The bulk (86·7%) of the recovered radioactivity was eluted in the 2% and 4% (v/v) diethyl ether in light petroleum fractions (fractions 2 and 3 respectively). Of the other fractions, the 15% (v/v) diethyl ether in light petroleum fraction (fraction 6) contained the most radioactivity (4·3%). Interfering substances were removed from these three fractions by further purification on 10 g columns of Decalso F to remove vitamin A and sterols. Thin-layer chromatography then showed that the bulk of the radioactive material in fractions 2 and 3 was α-tocopherol (R_f 0·75) and that the radioactive material in fraction 6 was α-tocopherolquinone (R_f 0·43). This was confirmed by the ultraviolet absorption spectrums of these fractions. Paper chromatography of fractions 2 and 3 revealed two radioactive components: one was α-tocopherol and accounted for 71% and 62% of the recovered radioactivity in these fractions; the other component corresponded in chromatographic behaviour to compounds found by Alaupović *et al.* (1961) and by Draper *et al.* (1962) and accounted for 12% and 15% of the radioactivity in the purified fractions 2 and 3, respectively. The latter compound was observed on paper chromatograms with an R_f close to 1·00 in the system 1% (v/v) acetone in light petroleum and an R_f close to zero in the reversed phase system developed with 95% (v/v) aqueous ethanol.

Spleen analysis

Table 1 shows that the lipids of the spleen became relatively highly labelled, as assessed by counts min g lipid, after rats were fed with [5-*Me*-^{14}C]-α-tocopherol. To establish the nature of the radioactive material, spleens were obtained from forty-six female rats killed 24 h after the administration by stomach tube of [5-*Me*-^{14}C]-α-tocopherol, and the chloroform-soluble lipids were extracted by the technique used to extract liver lipids. The pooled extracts were subdivided into an acetone-soluble fraction, containing 91% of the total lipid radioactivity, and an acetone-insoluble fraction, which was discarded. The acetone-soluble lipid was chromatographed on

acid-washed alumina, and radioactive fractions were purified on 10 g columns of Decalso F and by chromatography on thin layers of silica gel and zinc carbonate-impregnated papers, as described previously. Of the total radioactivity recovered from the alumina column, the bulk (74%) was in the fraction eluted by 4% (v/v) of diethyl ether in light petroleum. When measured portions of this fraction were chromatographed on thin layers of silica gel and on zinc carbonate-impregnated papers, only one radioactive compound was found. This was shown to be α-tocopherol by its ultraviolet absorption spectrum and by its chromatographic behaviour. The fraction eluted from alumina column by 15% (v/v) diethyl ether in light petroleum contained 12% of the recovered radioactivity, and this fraction clearly showed the ultraviolet absorption spectrum of α-tocopherolquinone; when chromatographed on thin layers of silica gel developed with 15% (v/v) of ethyl acetate in benzene, this radioactive material had the same R_f (0·57) as pure synthetic α-tocopherolquinone.

DISCUSSION

The pattern of distribution of radioactivity in the tissues of rats given [5-*Me*-^{14}C]-α-tocopherol orally was like that found by Wiss, Bunnell & Gloor (1962), who used [8-*Me*-^{14}C]-α-tocopheryl acetate in similar experiments. Thus spleen and adrenal tissue lipids reached the highest levels of radioactivity, with liver and kidney lipids also reaching relatively high levels. However, testis lipids, from an organ with a known requirement for vitamin E, were relatively low in radioactivity. Scharpenseel (1959) has shown that the high uptakes of radioactivity found in the adrenal and spleen are not specific to vitamin E or its metabolites. He gave tritiated vitamin A acetate to vitamin A-deficient rats and showed the highest uptake of radioactivity to be in the spleen, adrenals and gonads. Little is known about the role of the adrenals and the reticulo-endothelial system, especially the spleen, in lipid metabolism. In common with other parts of the reticulo-endothelial system, such as the Kupffer cells of the liver, the spleen has the capacity to remove lipids from the blood, which may explain the relatively high uptake of fat-soluble vitamins by the spleen.

Sternberg & Pascoe-Dawson (1959) found a second maximum of radioactivity in the stomach of rats 24 h after oral administration of [5-*Me*-^{14}C]-α-tocopheryl succinate. They attributed this phenomenon to the secretion of radioactive tocopherol or some metabolite into the lumen of the gut. Krishnamurthy & Bieri (1963) also reported a second increase in the radioactivity of the rat stomach and its contents after the ingestion of radioactive α-tocopherol. In both studies the second increase occurred 16–24 h after feeding, which suggests that it may have been caused by coprophagy. Sternberg & Pascoe-Dawson (1959) do not record that they prevented coprophagy. Krishnamurthy & Bieri used tail cups in some experiments, but it is not clear that the animals in which the second peak in ^{14}C activity was observed were so fitted. In our study, tail cups were fitted to rats kept alive longer than 12 h after the ingestion of a radioactive dose, and no second maximum was observed (Table 1).

Simon, Gross & Milhorat (1956) showed that tocopherol or some metabolite is secreted into the gut. They found 50–60% of the total radioactivity of an intra-

venous dose of [^{14}C]-α-tocopheryl succinate in the faeces of rabbits, previously deficient in vitamin E, over a period of 20 days. The maximum excretion occurred on the 4th day, when 7% of the administered radioactivity was found. Klatskin & Molander (1952) and Johnson (1956) suggested that the bile is a route for the excretion of α-tocopherol. Bile collected for 1 h before slaughter 3, 6, 12 and 24 h after oral administration of [^{14}C]-α-tocopherol contained only insignificant amounts of ^{14}C (Table 2). Schmandke & Proll (1964) found 2·4% of an intravenous injection of 5 mg α-tocopherol in the bile of rats in the subsequent 96 h; approximately 0·7% of the dose was found in the first 24 h. The larger dose, as well as the more direct route of administration, intravenous injection, led to greater excretion of tocopherol in the bile, as might be expected, but again these amounts were insufficient to explain the levels of tocopherol which have been found in the faeces.

In the liver and spleen cells the radioactivity from orally administered [5-*Me*-^{14}C]-α-tocopherol was associated mainly with the structural components. In the spleen most of the radioactivity found was associated with the mitochondria (60%), but in the liver a greater proportion of the recovered radioactivity was found in the microsomal fraction (44%) (Table 3).

No evidence was found for the presence in rat tissues of tocopheronic acid or its γ-lactone, found in the urine of rabbits and human subjects by Simon, Gross & Milhorat (1956) and Simon, Elsengart, Sundheim & Milhorat (1956). Nor was there any indication that α-tocopherol gave rise to a quinone, 2(farnesylfarnesylgeranylgeranyl)-3,5,6 trimethyl benzoquinone, $E_{2\,50}$, reported by Martius & Fürer (1963) to be a metabolite of α-tocopherol in the rat, chicken and earthworm. In our study, after administration of ^{14}C-labelled α-tocopherol to adequately fed rats and subsequent analysis of their spleen and liver lipids, it became clear that the bulk of the radioactivity within these lipids was in the form of α-tocopherol. After alumina chromatography of the spleen lipids, the bulk of the recovered radioactivity (55%) was shown to be α-tocopherol and 12% was shown to be α-tocopherolquinone. When the liver lipids were chromatographed on alumina, 87% of the recovered radioactivity was found in two adjacent fractions. Analyses of these two fractions showed that 60–70% of the recovered radioactive material in these fractions was α-tocopherol and that 12–15% of the radioactivity was associated with a compound having the chromatographic properties of the compound O found by Alaupović *et al.* (1961). Another fraction of the rat liver lipid contained 4% of the total recovered radioactivity and was shown to contain ^{14}C-labelled α-tocopherolquinone.

These results suggest that, in the livers of adequately fed rats, the formation of α-tocopherolquinone and compound O from α-tocopherol is not as extensive as in vitamin E-deficient livers (Draper *et al.* 1962). Our study supports the earlier evidence that α-tocopherolquinone and 'compound O' are normal metabolites of α-tocopherol in the rat, although the compounds can also arise as artifacts under certain conditions. These experiments, on rats not subjected to any nutritional deficiency, show that the greater part of α-tocopherol absorbed appears to remain intact in the tissues for some time.

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Tocopherol vs. Tocopherol Acetate as a "Sparer" of Vitamin A.

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The early observation[1] that materials containing vitamin E delayed the auto-oxidative destruction of carotene and vitamin A in fatty food mixtures was explained when the tocopherols were shown to be antioxidants.[2] That such protection may augment and conserve the vitamin A stores of an animal was first demonstrated by Moore[3] and has been confirmed repeatedly, as detailed in several reviews and some recent reports.[4]

The evidence suggests that inhibited oxidation in or near the alimentary tract[5] accounts for the survival of carotene and vitamin A. Recently,[6] this protective action appeared to be confined to preserving the store of vitamin A already deposited in the liver. The various forms of vitamin A behave differently. The results are modified by the form and method of administering the preparations of vitamin A[7] and vitamin E[8]. Other compounds having possible stabilizing effects[9] may be present and effective.

Several reports have stated or implied that the stabilizing action of the tocopherols is not limited to the alimentary tract but extends to the tissues of rats.[8,10] This was not demonstrable in rabbits.[11] Some information on this question might emerge from experiments with the esters of α-tocopherol, among them the acetate, which is also an oil, and is a dependable source of vitamin E, perhaps because it is not auto-oxidizable and has no antioxygenic action. Presumably, α-tocopherol acetate is hydrolyzed in the intestinal tract and the free alcohol should therefore be available in the tissues in quantities undiminished by having provided prior stabilization in the food.

The only pertinent observation on the acetate appears to be that of Bacharach,[12] to the effect that only at high levels of feeding and for an extended period (60 days) was any conservation of vitamin A demonstrable. A comparison of α-tocopherol and its acetate was therefore undertaken, to assess the relative protective value of each by simple experiments patterned after the U.S.P. bio-

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for vitamin A.

Experimental. d,l,α-tocopherol acetate. Rats of the Sprague-Dawley strain were given a stock diet until they weighed 40-50 g. Half of them were then transferred to a diet deficient in both vitamins A and E and having the following percentual composition: Vitamin-free casein, 20%; glucose, 67.5%; salt mixture,[13] 2.5%; ground cellophane, 3%; lard, 2%; yeast, 5%; viosterol (in peanut oil), 40 drops per kg. The other half were given the same diet except that hydrogenated vegetable fat (Spry) replaced the lard and 17.5 μg of *d,l,α*-tocopherol acetate* were added per kg of diet. Based on food consumption each animal received 70-80 γ of α-tocopherol acetate and 4-5 γ of tocopherols daily. In a second series the amount of α-tocopherol acetate added to the diet was 10 mg per kg. giving a daily intake of about 700 γ.

At the end of the deprivation period of 3-4 weeks when xerophthalmia or decline in weight or both became evident, the animals were properly grouped and vitamin A containing oils were administered daily, orally, by syringe with bent blunt needle. The standard U.S.P. Reference Oil No. 2 and the several shark liver oils† to be tested were diluted with cottonseed oil‡ to contain 2 I.U. of vitamin A per 0.1 cc (Reference Oil) or per 0.05 cc (liver oils). The oils were kept in the cold and new dilutions were made up fresh each week.

In Table I are the average figures obtained from these two series of animals. They indicate surprising and certainly acceptable uniformity in the times required for the development and cure of the symptoms of deficiency, whether α-tocopherol acetate was present in the diet or not. The weight gains on the reference oil were only half as great as those on the test oils, and the cure of xerophthalmia was also slower. This was doubtless due to the fact that this oil, as was reported later,[14] contained 30% less vitamin A than indicated. This figure was subsequently verified by the Carr-Price method against a standard vitamin A capsule kindly supplied by Distillation Products, Inc.

The vitamin A potency of the fish oils as indicated by the anti-xerophthalmic data agreed well with that of the chemical assays provided (Carr-Price, Rosenthal, Beckman). With the figures for gain in weight, the agreement was unsatisfactory, largely because of the unsuspected inferiority of the reference oil, and the consequent lack of a comparable standard dose-level.

d,l,α-tocopherol. The tests with the alcohol, made some time after those just described, differed from the earlier ones in several minor respects. Drisdol was used in place of viosterol as a source of vitamin D. Neither α-tocopherol nor vegetable oil was added to the basal diet of the experimental animals; α-tocopherol§ was given to them by mouth during the assay period only and in the same diluting olive oil which also contained the 2 I.U. of the vitamin A. The control animals received their vitamin A in olive oil. The sources of vitamin A were limited to the two reference oils U.S.P. Nos. 2 and 3 (the former after several years' storage in a dark ice-box). Finally, in lieu of a chemical determination of the liver stores of vitamin A, which at the moment could not be made on the necessary micro-scale, the survival time was determined after the close of the assay

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† Generously supplied by Hoffman La Roche, Inc. We are indebted to Dr. J. Murray Luck for samples of these oils and the results of chemical tests on them, on the basis of which they were selected for the bio-assays.

‡ Cottonseed oil was used designedly, if not solely, to stabilize the vitamin A in the oils before absorption. During the assay period, therefore, the vitamin E deficient animals on the test and reference oils received daily 20 and 40 γ of tocopherols, respectively; those on the low level dietary α tocopherol acetate had a 20-50% increase in the potential vitamin E intake, those on the high level, 3.7% increase. The data indicate that cottonseed oil was inconsequential in its effect on final results and that it may have accomplished its purpose.

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§ Kindly supplied by Merck and Company.

TABLE I.
Influence of α-Tocopherol Acetate on Bio-assay of Vit. A.
+E animals had 12.5 mg *d,l*-α-tocopherol acetate/kg of diet.

Source of curative Vit. A		Avg body wts at depletion, g	Avg gain in assay period, g	Xerophthalmia: Appeared in depletion period at avg days	Xerophthalmia: Cured in assay period at avg days
Refs. Oil 2	—E	84 (6)*	28 (1)†	23	22
Refs. Oil 2	+E	79 (6)	27 (1)	22	20
Oil 3	—E	74 (5)	38 (2)	23	21
Oil 3	+E	80 (8)	52	23	16
Oil 4	—E	80 (8)	46	24	18
Oil 4	+E	80 (7)	46	23	16
Oil 5	—E	80 (8)	47	23	19
Oil 5	+E	82 (7)	44 (1)	23	16
+E animals had 100 mg *d,l*-α-Tocopherol acetate/kg of diet.					
Refs. Oil 2	—E	76 (6)	25 (1)	28	17
Refs. Oil 2	+E	78 (6)	33 (1)	22	18
Oil 7	—E	76 (8)	65	23	11
Oil 7	+E	74 (7)	54	22	15
Oil 11	—E	75 (5)	50 (3)	25	10
Oil 11	+E	76 (7)	54	23	11
Oil 14c	—E	72 (6)	57 (1)	25	10
Oil 14c	+E	72 (7)	53 (1)	22	12

* No. of animals.
† No. of animals which died during the period and not included in the average.

TABLE II.
Influence of α-Tocopherol on Bio-assay of Vitamin A.

Reference oil and amt. of tocopherol, (mg)	Avg body wt at depletion, g	Gain in assay period, g	Xerophthalmia: Appeared in depletion period at avg days	Xerophthalmia: Cured in assay period at avg days	Avg survival in post-assay depletion, days
2 .0	85 (8)*	3	29	13	11
2 .5	91 (6)	17 (2)†	31	12	19
2 1.5	91 (6)	11 (2)	29	13	19
3 .0	90 (5)	4 (4)	30	16	14
3 .5	80 (8)	25	28	15	19
3 1.5	87 (7)	13 (2)	34	10	19
2 .0	80 (7)	11 (1)	27	11	17
2 .3	85 (5)	24 (3)	28	6	24
3 .0	82 (8)	24	27	7	15
3 .7	78 (7)	38 (1)	27	8	28

* No. of animals.
† No. of animals that died during the assay period and not included in the average.

period when the administration of supplements ceased. Two series of observations were made with each of the reference oils: in the first, 0.5 and 1.5 mg of α-tocopherol were administered with each oil, in the second 0.3 mg with oil No. 2, and 0.7 mg with oil No. 3.

Despite the unexplained high mortality in some of the lots, the data in Table II show that the survival in the post-assay depletion period was prolonged by α-tocopherol given in the assay period: (2) there was an optimum α-tocopherol dosage, perhaps 0.5-0.7 mg daily, beyond which the effectiveness of the vitamin A dosage decreased, as indicated by gains in weight in the assay period. Both of these findings are confirmatory of earlier reports[5] and point to an antioxygenic rather than a biological function of α-tocopherol. The stabilization of fats by α-tocopherol *in vitro* also demonstrates an optimum concen-

...ation: in the case of lard, this is between ...% and 0.10% of α-tocopherol.[15] Very recently, in similar experiments,[16] there is a suggestion that 0.5 mg α-tocopherol is an optimum dosage for the storage of vitamin A in the liver: the effect on vitamin A storage in the kidney was irregular. The liver stores of vitamin A from administered β-carotene were diminished by the larger doses of α-tocopherol and kidney storage was again irregular.

Unfortunately, no indication was secured as to the vitamin A stores of our animals on α-tocopherol acetate, following the assay period. The larger of the two dosages, 0.6 mg per day, even though it was within the optimum range and extended over both depletion and assay periods, may have been insufficient: it can hardly have been excessive. In any case, there was no response. The stabilization which the acetate can provide for vitamin A in the alimentary tract is limited to the amount of α-tocopherol available from it in the interval between hydrolysis and absorption. Perhaps, as with triglycerides, complete hydrolysis of the ester is not necessary for absorption, in which case any protective action in the tissues would depend upon the amount absorbed and the rate of subsequent hydrolysis. Information is needed on the rate of hydrolysis and absorption of tocopherol esters. The possible antioxygenic role of tocopherols in the tissues remains to be clarified.

Summary. 1. In bio-assays of vitamin A, α-tocopherol, but not α-tocopherol acetate, increased the rate of gain in the assay period. In a post-assay depletion period, animals that received α-tocopherol during the assay period survived 30-80% longer than controls.

2. As judged by gain in weight in the assay period, there is an optimum dosage of α-tocopherol; animals receiving 1.5 mg per day gained less than those receiving 0.5 mg. This conforms to the antioxygenic behavior of α-tocopherol.

3. Some of the possible causes for the failure of α-tocopherol acetate to conserve vitamin A are briefly discussed.

[15] Golumbic, Calvin, *Oil and Soap*, 1943, **20**, 105.

[16] Johnson, R. M., and Baumann, C. A., *J. Biol. Chem.*, 1948, **173**, 811.

Company Code [redacted]
Sub. No. 0227 - Tocopherols Concentrate, Mixed
0230 - d-Alpha Tocopheryl Acetate Concentrate
Box 55 (Question 76)

FINAL

Subacute Toxicity of Alpha Tocopherol Concentrate and Alpha Tocopheryl Acetate Concentrate in Rats

Introduction

There is a need to know the safety of tocopherol concentrates. Data obtained previously showed that commercially methylated tocopherol concentrates derived from soybean oil fed at a dietary level of 12% (up to 3.06% tocopherols) support normal growth and food utilization in rats. This experiment was designed to confirm the previous observations on Alpha Tocopherol Concentrate and to provide data on Alpha Tocopheryl Acetate Concentrate.

The objective was to determine the subacute toxicity of Alpha Tocopherol Concentrate and Alpha Tocopheryl Acetate Concentrate at high dosage levels in rats.

Experimental

The fats and tocopherol concentrates evaluated in this subacute toxicity feeding trial are described in detail as follows:

1. Deodorized refined cottonseed oil (Wesson Oil).
2. Alpha Tocopherol Concentrate (255 mg total tocopherols/g)
3. Alpha Tocopherol Acetate Concentrate (200 mg tocopheryl acetate/g)

The experimental design involved a control group fed ground Purina Dog Chow, a control group fed 1 g of Wesson Oil per day and two experimental groups fed the test materials at levels of 1 g/day. The supplements were fed in 4 g of ground dog chow five days a week. The rats were deprived of

food overnight five days a week to insure consumption of the fat supplement each morning. The fat supplements were made up in 1 kg batches and stored in the cold room to avoid deterioration.

Four groups of six 5 week old male rats each were maintained on ground Purina Dog Chow plus the above supplements for 105 days. Animals were weighed twice a week prior to the morning feeding of supplements.

Results

The growth responses observed are recorded in Table I and in Figure 1. All rats grew normally and no statistically significant differences at the 5% level were observed between group responses. No adverse effects of the experimental diets were observed during the experiment.

Discussion

Results of this experiment indicate that both the Alpha Tocopherol and Alpha Tocopheryl Acetate Concentrates fed at a level of 1 g/day/rat supported normal growth of male rats. The results further indicate that dietary levels of 255 mg/day of total tocopherols or 200 mg/day of tocopheryl acetate were innocuous when fed to rats for 105 days. These results confirmed the results of an earlier (unpublished) report that an estimated daily intake of 400 mg of tocopherol/rat is innocuous.

A dose of 1 g/day for a 167 g rat is equivalent to a dose of 420 g of tocopherol concentrate per day for a 70 kilo man. This is equivalent to a daily dose of 108 g/day of total tocopherols or 82 g/day of tocopheryl

acetate for the Alpha Tocopherol and Alpha Tocopherol Acetate Concentrates, respectively. Thus, Tocopherol Concentrates are innocuous in rats at levels about 10,000 times the NAS-NRC Recommended Daily Dietary Allowances of 30 I.U./day for an adult male.

Conclusions

It is concluded that daily doses of 1 g of Alpha Tocopherol Concentrate (255 mg total tocopherols/day) or Alpha Tocopheryl Acetate Concentrate (200 mg tocopheryl acetate/day) support normal growth in rats.

Company Code [redacted]
Sub. No. 0227 - Tocopherols Concentrate, Mixed
0230 - d-Alpha Tocopheryl Acetate Concentrate
Box 55 (Question 7b)

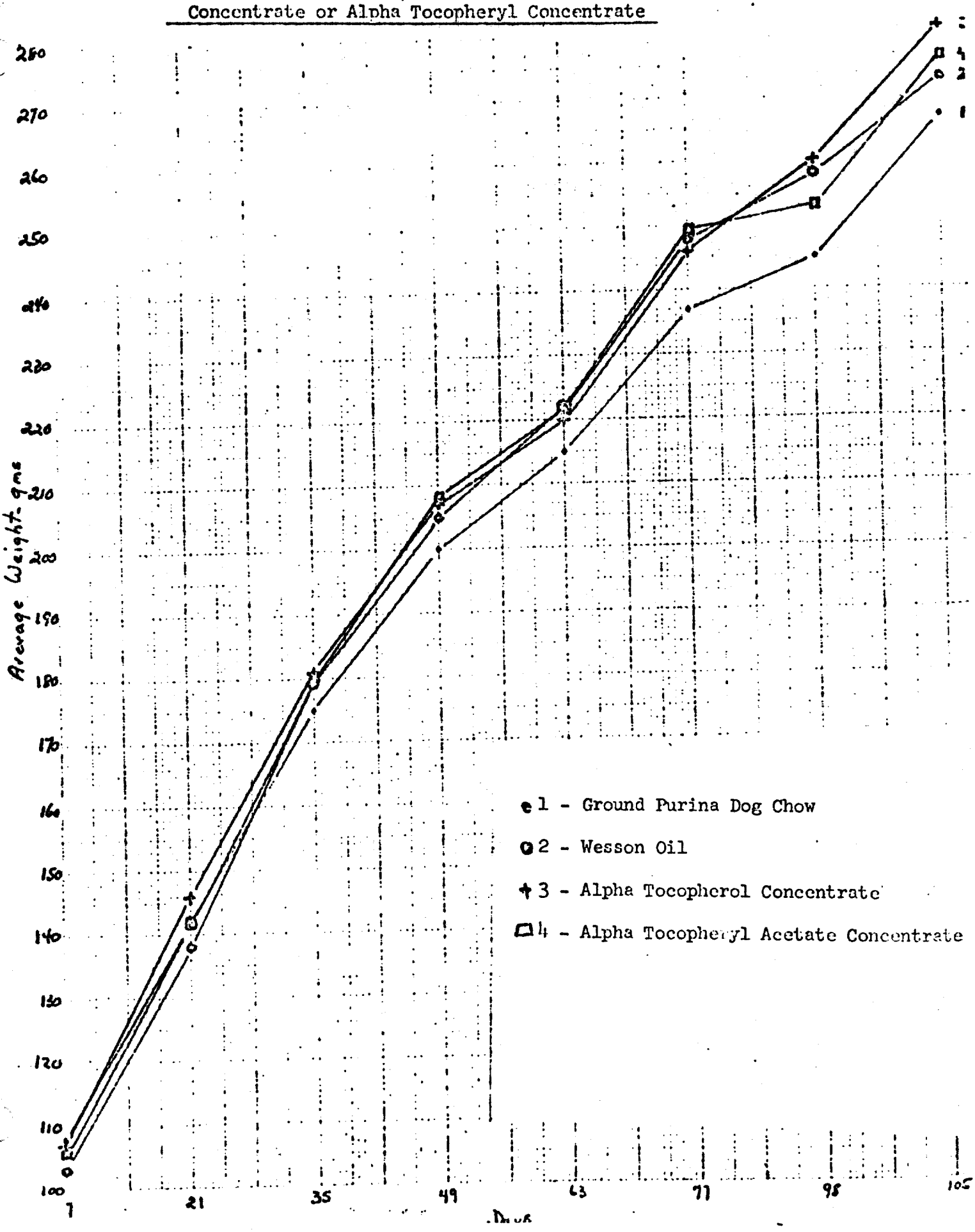
Table I

Growth of Rats Fed 1 g/day of Alpha Tocopherol Concentrate or Alpha Tocopheryl Acetate Concentrate

Group	Diet	Supplement+	Average weight (g) at these day intervals								Average Total Gain in Wt. (g) ± S.E.
			7	21	35	49	63	77	98	105	
1	Purina Dog Chow, ground	--	107.6	141.7	174.7	199.8	215.3	236.8	245.3	266.5	166.4 ± 7.8
2	" "	Wesson Oil	103.0	137.7	178.7	204.5	222.2	247.8	257.7	273.4	175.8 ± 10.7
3	" "	Alpha Tocopherol Concentrate	107.3	145.7	180.8	206.7	219.7	245.8	260.0	280.8	181.8 ± 4.0
4	" "	Alpha Tocopheryl Acetate Concentrate	105.6	141.7	180.3	208.0	222.0	249.0	253.2	276.3	178.5 ± 11.5

+1 gram of fat per 4 grams of ground Purina Dog Chow - 5 days per week

Growth of Rats Fed 1 g/day of Alpha Tocopherol Concentrate or Alpha Tocopheryl Concentrate



The fertility experiment was carried out with four groups of rats from the toxicity experiment; both control groups and the two lower levels of tocopheryl acetate supplementation. The rats were mated in groups of three. Each of these groups consisted of a male, a control female and a tocopheryl acetate supplemented female. Males from the colony with satisfactory previous mating records were used. The mating period was approximately two weeks. Supplements of tocopheryl acetate were continued until all control rats had delivered their young.

Results

The growth responses observed are recorded in Table I and Figure 1. The growth of rats receiving the d-Alpha Tocopheryl Acetate Concentrate at a level of 0.50 g/day and below was not significantly different from that of control rats. At the highest level of tocopheryl acetate (1.0 g/day), growth was depressed and three rats died within two weeks. The food consumption and utilization data are summarized in Table II. Rats fed d-Alpha Tocopheryl Acetate Concentrate at levels of 0.50 g/day and below had feed efficiencies comparable to that of the olive oil supplemented group but lower than the unsupplemented control group. At the highest level of tocopheryl acetate supplementation (1 g/day), food consumption was greatly depressed.

The results of the reproduction study are summarized in Table III. d-Alpha Tocopheryl Acetate Concentrate did not inhibit fertility or number of young born per litter. The viability of young from rats given tocopheryl acetate appeared to be better than that for young from control rats.

Company Code [redacted]
Sub. No. 0230 - d-Alpha Tocopheryl Acetate Concentrate
Box 55 (Question 7b)

Subacute Toxicity and Effects on Reproduction of d-Alpha Tocopheryl Acetate in Rats

Introduction

There is a need to know the safety of tocopherol concentrates. Data obtained previously showed that d-Alpha Tocopheryl Acetate Concentrate (200 mg tocopheryl acetate/day) supported normal growth in rats. This experiment was designed to confirm and extend the toxicity data on tocopheryl acetate and to provide data on its effect on reproduction.

The objective was to determine the subacute toxicity and effect on reproduction of d-Alpha Tocopheryl Acetate Concentrate at high dosage levels in rats.

Experimental

The experimental design for the toxicity test included an unsupplemented control group, a control group supplemented with 1.0 ml of olive oil per day and four experimental groups supplemented with 0.125, 0.25, 0.50 and 1.0 g of d-Alpha Tocopheryl Acetate Concentrate per day. The three lower levels of tocopheryl acetate supplement were administered as dilutions in olive oil to provide a volume of 1.0 ml daily. All supplements were administered orally by syringe five days per week. All animals were fed on Stock Diet I ad lib except that diet was withdrawn for the four-hour period prior to supplementation.

Six groups of five weanling female rats each were maintained on the toxicity experiment for 14 weeks. Animals were weighed twice a week and food consumption was recorded daily for 10 weeks.

Company Code [redacted]
Sub. No. 0230 - d-Alpha Tocopheryl Acetate Concentrate
Box 55 (Question 7b)

Subacute Toxicity and Effects on Reproduction of d-Alpha Tocopheryl Acetate in Rats

Introduction

There is a need to know the safety of tocopherol concentrates. Data obtained previously showed that d-Alpha Tocopheryl Acetate Concentrate (200 mg tocopheryl acetate/day) supported normal growth in rats. This experiment was designed to confirm and extend the toxicity data on tocopheryl acetate and to provide data on its effect on reproduction.

The objective was to determine the subacute toxicity and effect on reproduction of d-Alpha Tocopheryl Acetate Concentrate at high dosage levels in rats.

Experimental

The experimental design for the toxicity test included an unsupplemented control group, a control group supplemented with 1.0 ml of olive oil per day and four experimental groups supplemented with 0.125, 0.25, 0.50 and 1.0 g of d-Alpha Tocopheryl Acetate Concentrate per day. The three lower levels of tocopheryl acetate supplement were administered as dilutions in olive oil to provide a volume of 1.0 ml daily. All supplements were administered orally by syringe five days per week. All animals were fed on Stock Diet I ad lib except that diet was withdrawn for the four-hour period prior to supplementation.

Six groups of five weanling female rats each were maintained on the toxicity experiment for 14 weeks. Animals were weighed twice a week and food consumption was recorded daily for 10 weeks.

The fertility experiment was carried out with four groups of rats from the toxicity experiment; both control groups and the two lower levels of tocopheryl acetate supplementation. The rats were mated in groups of three. Each of these groups consisted of a male, a control female and a tocopheryl acetate supplemented female. Males from the colony with satisfactory previous mating records were used. The mating period was approximately two weeks. Supplements of tocopheryl acetate were continued until all control rats had delivered their young.

Results

The growth responses observed are recorded in Table I and Figure 1. The growth of rats receiving the d-Alpha Tocopheryl Acetate Concentrate at a level of 0.50 g/day and below was not significantly different from that of control rats. At the highest level of tocopheryl acetate (1.0 g/day), growth was depressed and three rats died within two weeks. The food consumption and utilization data are summarized in Table II. Rats fed d-Alpha Tocopheryl Acetate Concentrate at levels of 0.50 g/day and below had feed efficiencies comparable to that of the olive oil supplemented group but lower than the unsupplemented control group. At the highest level of tocopheryl acetate supplementation (1 g/day), food consumption was greatly depressed.

The results of the reproduction study are summarized in Table III. d-Alpha Tocopheryl Acetate Concentrate did not inhibit fertility or number of young born per litter. The viability of young from rats given tocopheryl acetate appeared to be better than that for young from control rats.

Discussion

The results of this experiment indicated that levels of d-Alpha Tocopheryl Acetate Concentrate at levels of 0.50 g/day and below supported normal growth of female rats. The highest level of tocopheryl acetate (1.0 g/day) caused severe growth depression and mortality. These data indicate that d-Alpha Tocopheryl Acetate Concentrate is not toxic at a level of 0.50 g/50 g rat/day but is toxic at a level of 1.0 g/50 g rat/day. Daily doses up to 10 g/kg body wt./day are innocuous in weanling rats.

Reproduction of rats was unaffected by d-Alpha Tocopheryl Acetate Concentrate at dosage levels as high as 0.25 g/rat/day. Viability of the progeny may have been enhanced by tocopheryl acetate supplementation. The average weaning weight of the progeny from rats receiving tocopheryl acetate supplementation was lower than from the control group. This is consistant with the increased size of litters for the tocopheryl acetate supplemented female rats.

Conclusion

It is concluded that levels of 0.50 g/day and below of d-Alpha Tocopheryl Acetate Concentrate support normal growth of rats. Mortality and growth depression were observed at the 1.0 g/day level. Reproduction was unaffected by d-Alpha Tocopheryl Acetate Concentrate at dosage levels as high as 0.25 g/day.

[illegible] Code [illegible]
S[illegible] No. 0230 - d-Alpha Tocopheryl Acetate Concentrate
Box 55 (Question 7b)

Table I

Growth of Rats Fed d-Alpha Tocopheryl Acetate Concentrate

Group	Supplement 1.0 ml/5x/wk.	Average Weight (g) at Weekly Intervals									
		0	1	2	3	4	6	8	10	12	14
1	--	45.4	80.5	111.8	135.0	151.8	180.0	198.0	210.8	223.0	229.3
2	Olive Oil	46.6	74.2	101.2	126.0	144.6	166.0	180.4	188.6	204.0	211.8
3	0.125 g[a]	45.2	75.0	102.0	126.4	141.8	161.2	173.4	187.8	197.4	204.6
4	0.250 g[a]	46.2	75.0	103.2	125.6	142.4	170.2	178.4	192.4	204.4	211.8
5	0.500 g[a]	46.8	74.8	103.4	121.8	139.8	163.0	179.6	190.4	200.8	206.4
6	1.0 g[a]	46.2	48.7	72.5	78.5	88.0[b]	--	--	--	--	--

[a]Supplement of d-Alpha Tocopheryl Acetate Concentrate in grams.

[b]Experimental group discontinued.

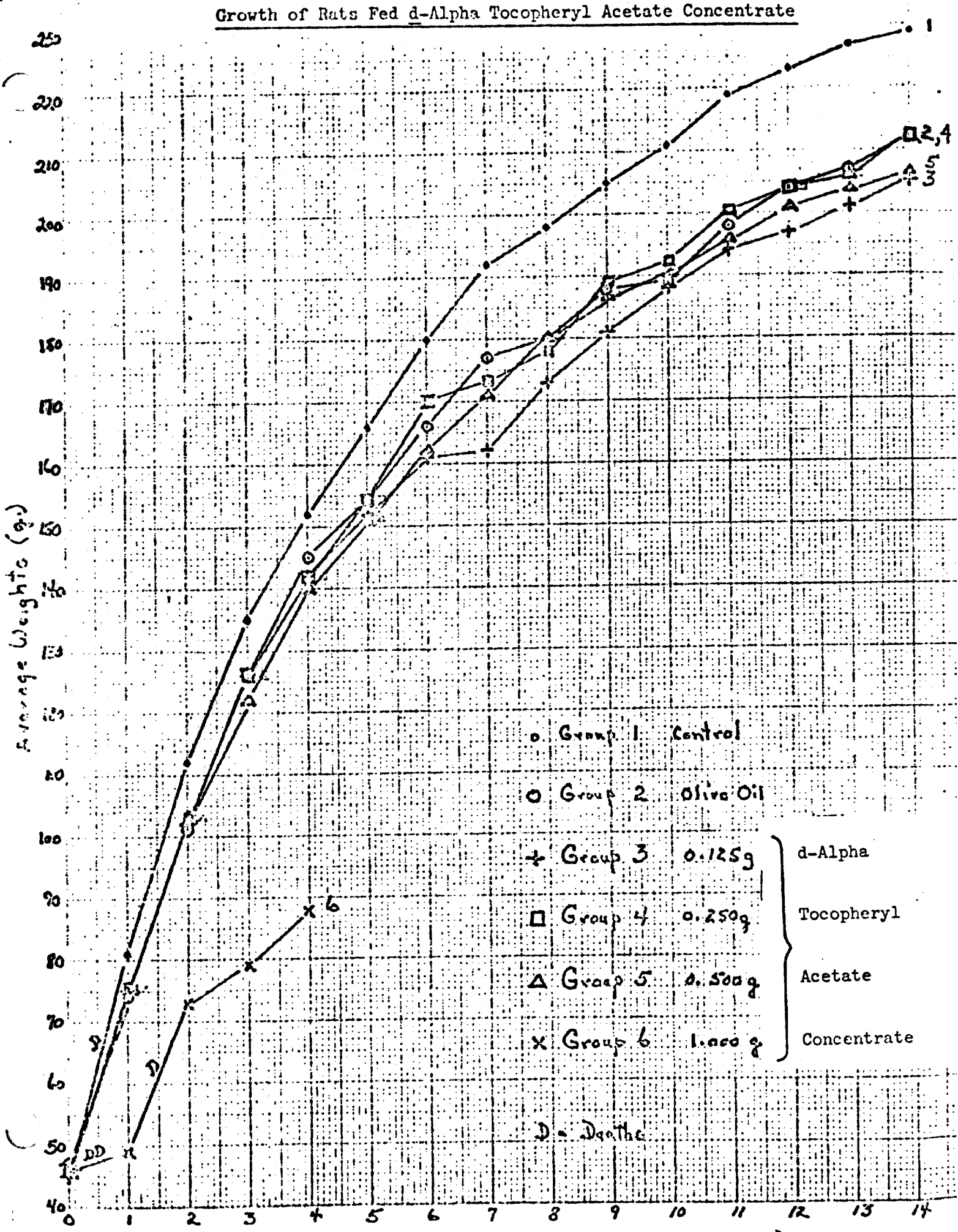
C[redacted]any Code [redacted]
Sub. No. 0230 - d-Alpha Tocopheryl Acetate Concentrate
Box 55 (Question 7b)

Table III

Effects on Reproduction of Rats Fed d-Alpha Tocopheryl Acetate Concentrate

Group	Supplement 1.0 ml/5x/wk.	Ave. No. per Litter	Total No. Weaned	Ave. No. Weaned	Sex Ratio		Ave. Weight at Weaning (g)
					Males	Females	
1	--	8.8	31	7.8	20	11	43.0
2	Olive Oil	9.0	39	7.8	20	19	42.2
3	d-Alpha Tocopheryl Acetate Concentrate 0.125 g	10.2	49	9.8	25	24	34.6
4	d-Alpha Tocopheryl Acetate Concentrate 0.250 g	9.2	30	10.0	14	16	32.7

Growth of Rats Fed d-Alpha Tocopheryl Acetate Concentrate



Company Code [redacted]
Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Reports on Unpublished Studies

Subject: Feeding Studies in Chicks of d-Alpha Tocopheryl Acetate and Three Feed Grade Alpha-Tocopheryl Acetates

Date: September 4, 1968

Summary

Chicks were fed for 21 days on a control ration or the control ration supplemented with either 500 or 5,000 I.U. per kg with either d-alpha-tocopheryl acetate or one of three commercial feed grade synthetic alpha-tocopheryl acetates (A, B, C). There was no significant difference in growth between preparations at the 500 I.U./kg level. At the 5,000 I.U./kg level, growth was significantly different between preparations with the group fed d-alpha-tocopheryl acetate showing the lowest gain. Kidney and liver weights showed no significant differences attributable to dose levels or preparations.

Introduction

The effect of feeding chicks d-alpha-tocopheryl acetate or commercial synthetic alpha-tocopheryl acetate on growth, liver and kidney weights, and gross pathology were determined when fed in a commercial chick starter ration at levels of 500 and 5,000 I.U./kg for 21 days.

Experimental

Diets

Nine groups of chicks were fed for three weeks either Purina Chick Starter, or the Purina Chick Starter with 500 or 5,000 I.U. of either d-alpha-tocopheryl acetate commercial feed grade synthetic alpha-tocopheryl acetate. All treatments were fed in duplicate. Diet composition is

Animals

Two hundred and fifty day old White Mountain cross cockerels were purchased from Hubbard Farms, Walpole, New Hampshire. Upon arrival, all chicks were individually wing-banded and weighed. The smallest and largest chicks were discarded until 180 birds remained. Chicks were combined into 18 groups of 10 chicks each so that each group had the same average weight and approximately the same distribution of the various size chicks. Immediately after sorting, the chicks were placed in their respective pens with their rations already in the troughs.

The chicks were weighed at 0, 7, 11, 14, 18 and 21 days. At the termination of the assay, feed consumption was determined, chicks were sacrificed and examined for gross pathology. Livers and kidneys were removed and weighed.

Results

Treatment of Data

Individual weight gains and relative liver and kidney weights were submitted for analysis of variance.

Growth

Table II summarizes the growth response of chicks on the basal diet and two levels of d-alpha-tocopheryl acetate and three commercial feed grade synthetic alpha-tocopheryl acetates.

When the growth data was analyzed statistically, there was no significant difference between preparations at the low level. However,

there was a significant difference between preparations at the high level; the lowest gain being observed on the diet containing d-alpha-tocopheryl acetate.

"F" values for dose levels and preparations could not be determined from the data presented. The interaction between dose and preparation was significantly different.

Feed efficiency $\frac{(\text{Feed Consumed})}{(\text{Total Gain})}$ was determined at the conclusion of the feeding trial. This data did not reveal any trends for either preparations or levels compared with the control ration.

Gross Pathology and Organ Weights

At autopsy, chicks fed the control ration and those fed two levels of d-alpha-tocopheryl acetate and the three feed grade synthetic alpha-tocopheryl acetates showed no gross abnormalities. Livers and kidneys were removed from all chicks. Mean weights for these organs per 100 g body weight are presented in Table III.

Relative kidney weights indicated no significant difference for dose level, preparation or their interaction when subjected to analysis of variance.

Relative liver weights showed no significant differences for dose level or preparation but their interaction was significant.

Company ic [redacted]
Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Table I

Diet Composition

Ingredients	Control Diet			Test Groups					
	Group 1 & 18 g/kg	Group 2 & 14 g/kg	Group 3 & 15 g/kg	Group 4 & 16 g/kg	Group 5 & 17 g/kg	Group 6 & 10 g/kg	Group 7 & 11 g/kg	Group 8 & 12 g/kg	Group 9 & 13 g/kg
Chick Starter - no tocopherol	1000	999.63	996.30	999.41	994.08	999.5	999.6	993.98	939.8
d-Alpha-Tocopheryl Acetate	--	0.370	3.70	--	--	--	--	--	--
Commercial A Feed Grade Synthetic Alpha Tocopheryl Acetate	--	--	--	0.592	5.92	--	--	--	--
Commercial B Feed Grade Synthetic Alpha Tocopheryl Acetate	--	--	--	--	--	0.545	5.45	--	--
Commercial C Synthetic Alpha-Tocopheryl Acetate	--	--	--	--	--	--	--	6.02	60.20

Sub. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Table II

Growth

Pen No.	Ration	Level Fed IU/kg.	Weight (grams) (Average of two pens) 0 days	7 days	11 days	14 days	18 days	21 days	Mean Gain (g ± SE)
1-18	Control ration-chick starter - no tocopherol	--	45.4	113.2	174.1	234.8	330.4	412.4	366.0 ± 3.71
2-14	Chick starter + d-alpha-tocopheryl acetate	500	45.5	114.8	176.2	237.8	332.2	406.5	361.0 ± 11.3
3-15	Chick starter + d-alpha-tocopheryl acetate	5000	45.4	112.6	167.2	214.7	284.0	344.3	298.9 ± 2.56
4-16	Chick starter + Commerical A feed grade synthetic alpha-tocopheryl acetate	500	45.4	114.9	174.0	232.5	330.0	411.4	366.0 ± 13.31
5-17	Chick starter + Commerical A feed grade synthetic alpha-tocopheryl acetate	5000	45.6	108.9	159.6	211.4	298.0	367.1	321.1 ± 11.34
6-10	Chick starter + Commercial B feed grade synthetic alpha-tocopheryl acetate	500	45.2	107.9	163.0	216.2	311.9	394.7	349.5 ± 13.30
7-11	Chick starter + Commercial B feed grade synthetic alpha-tocopheryl acetate	5000	45.2	113.1	172.0	226.7	328.7	415.5	370.3 ± 10.77
8-12	Chick starter + Commerical C feed grade synthetic alpha-tocopheryl acetate	500	45.2	113.9	173.2	230.4	331.7	419.2	374.0 ± 10.61
9-13	Chick starter + Commerical C feed grade synthetic alpha-tocopheryl acetate	5000	45.2	108.8	166.0	217.1	315.6	395.2	351.1 + 15.10

Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Table III

Mean Organ Weights per 100 g Body Weight ± SE

Ration	Alpha-Tocopherol	Liver (grams)	Kidneys (grams)
Control - Chick Starter (Purina)	--	2.76 ± 0.013	0.81 ± 0.052
d-alpha-tocopheryl acetate	500	2.66 ± 0.050	0.97 ± 0.022
	5000	3.34 ± 0.074	0.99 ± 0.075
Commercial A Feed Grade Synthetic alpha-tocopheryl acetate	500	2.71 ± 0.075	0.96 ± 0.027
	5000	2.92 ± 0.038	0.92 ± 0.023
Commercial B Feed Grade Synthetic alpha-tocopheryl acetate	500	2.73 ± 0.050	0.93 ± 0.026
	5000	2.85 ± 0.060	0.93 ± 0.020
Commercial C Feed Grade Synthetic alpha-tocopheryl acetate	500	2.70 ± 0.060	0.96 ± 0.020
	5000	2.95 ± 0.069	0.96 ± 0.020

Company Code [redacted]
Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Reports on Unpublished Studies

Subject: Feeding Trials on d- and alpha-Tocopheryl Acetate Preparations

Date: June 4, 1968

Summary

In an eight-week study, groups of male rats were fed a control diet or the same diet supplemented with 500 or 5000 IU/kg of either d-alpha-tocopheryl acetate or two commercial feed grade synthetic alpha-tocopheryl acetates (A and B) to ascertain their possible effects on growth, growth pathology and organ weights.

Growth of all rats supplemented with either natural or synthetic alpha-tocopheryl acetates was comparable to that of the control rats. No gross abnormalities were observed when rats were autopsied. The weights of kidneys, testes, and adrenals were comparable for all groups of rats. The liver weights showed significant variation between groups.

Introduction

The toxicity of high levels of d-alpha-tocopheryl acetate and natural and synthetic alpha-tocopherols have been determined. Fasted weanling female rats were orally fed d-alpha-tocopheryl acetate at levels of 0.125, 0.25, 0.5 and 1.0 grams five days per week for 14 weeks. Depressed growth and mortality was observed at the 1.0 gram level. The three lower levels of d-alpha-tocopheryl acetate had no effect on growth, food consumption and mortality. There was no effect on reproduction, lactation and weaning weights of young

when rats received 0.25 and 0.125 gram levels. A daily level of 0.5 grams of d-alpha-tocopheryl acetate was comparable to a dietary level of 3% or 40,000 IU/k In another (unpublished) study, no adverse effect on fertility, mortality, litte efficiency and viability and lactation of young was observed when mated female rats were fed orally 0.1 and 0.5 grams daily of d-alpha-tocopherol and dl-alpha-tocopherol throughout the gestation period.

In this experiment, the effect of d- and two synthetic alpha-tocopheryl acetate preparations on growth, organ weights and gross pathology of rats were determined when fed in the diet at 500 and 5000 IU/kg.

Experimental

Diets

Seven groups of rats were fed for eight weeks either the stock diet (Diet 1) or stock diet with 500 or 5000 IU/kg of either d-alpha-tocopheryl acetate or two synthetic feed grade synthetic alpha-tocopheryl acetates. These two levels of the tocopherol preparations were selected for the feeding tests in rats on the basis of results of previous work in this laboratory and on data estimating maximum levels used for humans, for turkey breeder rations, and for experimental cattle rations. Table I summarizes the composition of the respective diets.

Animals

Weanling male rats from Blue Spruce Farms, Altamount, New York, were fed the stock diet (Diet 1) for one week. During this period the rats were

weighed and examined regularly in order to eliminate those with poor growth and physical defects. The experimental animals which were selected from the remaining rats had a limited weight range (69 g to 91 g). Twelve rats were randomly distributed to each of the seven dietary groups.

All rats on the experiment were observed and weighed weekly. After eight weeks all groups were examined for gross pathology. The following organs were removed and weighed: liver, kidneys, adrenals and testes.

Results

Treatment of Data

The individual weight gains and liver and testes weights per 100 g body weight were submitted for statistical analysis. The results of the analysis of variance for these variables are summarized in Table II in which the F-values are tabulated for dose level, preparation and interaction.

Growth

The growth response of the rats on the basal diet and the two levels of the natural and synthetic alpha-tocopheryl acetates is summarized in Table III.

All rats which were fed Diet 1 supplemented with either the natural or synthetic alpha-tocopheryl acetates grew at a rate comparable to control rats on Diet 1. When these growth data were analyzed statistically by analysis of variance, no significant difference in either dose level, preparation or interaction between dose and tocopherol preparations was observed.

Gross Pathology and Organ Weight

No gross abnormalities were observed when control rats and those fed two levels of either d-alpha-tocopheryl acetate or synthetic alpha-tocopheryl acetates were autopsied after eight weeks on experiment. The liver, kidneys, adrenals and testes were removed from all rats and weighed. The mean organ weights on basis of 100 g body weight are summarized in Table IV.

No significant differences in weights of kidneys, testes and adrenals was observed. The analysis of variance for the testes weights indicated that the interaction between the dose levels and preparations of natural and synthetic alpha-tocopheryl acetates was significant at the 1% level of probability. We have no explanation for the significance of the interaction variance.

Inspection of the preparation means indicated that the mean liver weight of rats fed both levels of the Commercial B alpha-tocopheryl acetate (4.80) was similar to that of the control (4.85). The mean liver weight of rats fed both levels of d-alpha-tocopheryl acetate was less (4.61) and that for the Commercial A synthetic alpha-tocopheryl acetate approached atrophy (4.45). Analysis of variance indicated a significant difference between preparation mean liver weights at the 1% level of probability. We have no explanation for these observed significant differences in preparation means.

Company Code [redacted]
Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Table I

Diet Composition

Ingredients	Control Diet	Test Diets					
	Group 1 g/kg	Group 2 g/kg	Group 3 g/kg	Group 4 g/kg	Group 5 g/kg	Group 6 g/kg	Group 7 g/kg
Diet 1	1000	999.63	996.30	999.40	994.1	999.5	994.6
d-alpha-tocopheryl acetate	--	0.370	3.70	--	--	--	--
Commercial A dl-alpha-tocopheryl acetate	--	--	--	0.592	5.92	--	--
Commercial B dl-alpha-tocopheryl acetate	--	--	--	--	--	.545	5.45

Company Code [redacted]
Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Table II

F. Values from Analysis of Variance

		Variable	
Effect of	Growth	Liver Wt.	Testes Wt.
Dose Level	1.14	0.00021	--
Preparation	1.18	5.27	--
Interaction	1.33	1.01	5.60

Company Code [redacted]
Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Table III

Growth of Rats on Diet 1 and Diet 1 with Added d-alpha-Tocopheryl Acetate and Commercial Feed Grade dl-alpha-Tocopheryl Acetates

Diet	Mean Weight (g)						Mean Weight Gain g ± SE
	Initial	1 Week	2 Weeks	4 Weeks	6 Weeks	8 Weeks	
Group 1 Control Diet 1	77.0	113.6	150.8	232.3	293.8	354.9	277.9 ± 11.08
Group 2 d-alpha-tocopheryl acetate 500 I.U.	77.0	113.9	152.2	236.4	295.8	357.5	280.1 ± 10.92
Group 3 d-alpha-tocopheryl acetate 5000 I.U.	77.1	110.7	146.3	224.1	283.9	341.9	264.8 ± 9.85
Group 4 Commercial A Feed Grade dl-alpha-tocopheryl acetate 500 I.U.	75.6	112.8	147.6	224.5	285.7	340.4	264.8 ± 7.96
Group 5 Commercial A Feed Grade dl-alpha-tocopheryl acetate 5000 I.U.	76.1	114.0	154.4	235.8	285.7	350.7	274.6 ± 8.82
Group 6 Commercial B Feed Grade dl-alpha-tocopheryl acetate 500 I.U.	75.9	115.1	156.7	240.7	303.0	370.3	294.3 ± 11.76
Group 7 Commercial B Feed Grade dl-alpha-tocopheryl acetate 5000 I.U.	75.2	111.2	151.8	227.4	282.3	348.9	273.8 ± 9.70

Company Code [redacted]
Sub. No. 0228 - d-Alpha Tocopheryl Acetate
Box 55 (Question 7b)

Table IV

Mean Organ Weights per 100 g Body Weight ± SE

Diet	alpha-Tocopherol I.U./kg	Liver	Kidneys	Adrenals	Testes
Control					
Diet 1	--	4.85 ± 0.09	0.80 ± 0.011	0.015 ± 0.0011	0.94 ± 0.026
Test					
d-alpha-tocopheryl acetate	500	4.54 ± 0.13	0.82 ± 0.015	0.015 ± 0.0016	0.91 ± 0.011
	5000	4.68 ± 0.12	0.82 ± 0.022	0.015 ± 0.0014	1.00 ± 0.032
Commercial A dl-alpha-tocopheryl acetate	500	4.43 ± 0.13	0.84 ± 0.034	0.013 ± 0.0004	0.98 ± 0.018
	5000	4.46 ± 0.07	0.80 ± 0.022	0.014 ± 0.0006	0.95 ± 0.020
Commercial B dl-alpha-tocopheryl acetate	500	4.89 ± 0.09	0.80 ± 0.011	0.013 ± 0.0005	0.90 ± 0.049
	5000	4.72 ± 0.12	0.83 ± 0.013	0.014 ± 0.0006	1.02 ± 0.019

Company Code [redacted]
Sub. No. 0227 - Tocopherols Concentrate, Mixed
Box 55 (Question 7b)

Reports on Unpublished Studies

Subject: Effect of massive doses of natural and synthetic alpha-tocopherol and DPPD on reproduction of rats

Date: March 17, 1958

Summary - Massive doses (100 or 500 mg/day) of synthetic or natural alpha-tocopherol had no effect on female rate during gestation or lactation. In agreement with previous experiments, supplementation of pregnant female rats with 100 mg/day of DPPD prolonged the gestation period and caused maternal and total infant mortality.

The purpose of this experiment was to determine whether free alpha-tocopherol fed at high levels to pregnant rats would have the DPPD-like effect of prolonging the gestation period and causing death in some of the mothers and young. Also, natural and synthetic alpha-tocopherols were compared to see if massive doses administered during pregnancy showed differences in response.

Forty female rats were weaned during a two-week period (5/29/56 to 6/12/56); were maintained on vitamin E-free Diet #301 for 5 weeks; and then were transferred to stock diet #1 (7/6/56). When the rats had attained a 150 gram weight, they were mated over a period of 6 weeks (7/17/56 to 8/27/56). Insemination was determined by daily vaginal smearing.

Both tocopherol and N, N'-diphenyl-p-phenylenediamine (DPPD) supplements in olive oil were administered orally by dropper daily during the gestation

period (21 days). The following table indicates the groups and their respective supplements:

Group	No.	d-Alpha-Tocopherol mg/day	dl-Alpha-Tocopherol mg/day	DPPD mg/day
1	8	100	-	-
2	8	500	-	-
3	8	-	100	-
4	8	-	500	-
5	8	-	-	100

The droppers used for the tocopherols were calibrated for the mixtures which were considerably more viscous and heavy than olive oil. The DPPD was not completely soluble in olive oil, so the mixture was shaken before each feeding.

After the females were mated, they were weighed every four days during the gestation period. The progeny were weighed on the 3rd and 21st days after birth.

The attached table completely summarizes the results of the project.

The total mean weight gained during the gestation period was comparable for both natural and synthetic tocopherol supplemented rats. The 500 mg. levels of both tocopherols and the 100 mg level of DPPD produced slightly less weight gain than the 100 mg levels of the tocopherols. This was probably due to the small mean litter sizes for these groups which was established prior to supplementation.

The length of the gestation was not effected by the tocopherol supplements. DPPD extended the gestation period though not as much as was observed previously

in rats fed a vitamin E-free diet. All the mated females survived the gestation period except one supplemented with DPPD that died during the terminal stage of gestation.

The mean weights of the young at 3 and 21 days were comparable for all groups. The viability of the tocopherol supplemented rats was 80-90%, but the young from the DPPD rats did not survive the third day. The young from tocopherol-supplemented rats were raised to weaning with practically 100% survival.

Company Code [redacted]
Sub. No. 0227 - Tocopherols Concentrate, Mixed
Box 55 (Question 7b)

Table I

Summary

Effect of Massive Doses of Natural and Synthetic Alpha Tocopherol and DPPD Administered During Gestation Period on Reproduction and Lactation of Rats on Stock Diet

Group	No. Rats	Supplement[1]	Mean Gain in Wt. During Gestation g	Mean Length of Gestation Period days	Fertility Index[2]	Litter Efficiency[3] %	Mortality Index[4]	Mean Litter Size at Birth	Mean 3-Day Wt. g	Viability Index[5]	Mean 21-Day Wt. g	Lactation Index[6]
1	8	d-α-tocopherol 100 mg/day	101.7	22	8/8	100	0/8	10.2	7.0	50/61(82%)	37.2	50/50
2	7	d-α-tocopherol 500 mg/day	83.0	23	4/7	100	0/7	8.3	8.1	24/25(96%)	34.9	23/24
3	7	dl-α-tocopherol 100 mg/day	115.4	23	7/7	100	0/7	10.9	7.4	68/76(89%)	37.0	66/68
4	7	dl-α-tocopherol 500 mg/day	84.0	23	7/7	100	0/7	8.0	7.6	44/56(79%)	38.3	44/44
5	7	DPPD 100 mg/day	83.7	24	7/7	100	1/7	7.7	-	0/23(0%)	-	-

[1] dl-α-tocopherol (commercial)

[2] Fertility Index - ratio of females pregnant to females mated.

[3] Litter Efficiency - percentage of positive responses (one or more viable fetuses) to total number of animals with at least 4 implantations.

[4] Mortality Index - ratio of females that died to females mated.

[5] Viability Index - ratio of young alive at 3 days to young born.

[6] Lactation Index - ratio of young weaned to young alive at 3 days.

Company Code [redacted]
Sub. No. 0227 - Tocopherols Concentrate, Mixed
Box 55 (Question 7b)

Subacute Toxicity on Tocopherol Concentrates in Rats

Introduction

There is a need to know the safety of tocopherol concentrates derived from soybean oil. Two different samples of commercially methylated soybean oil concentrate were tested; a low potency tocopherol concentrate completely processed except that the final distillation step was omitted and a high potency alpha tocopherol concentrate. The edible fats; lard, hydrogenated vegetable shortening (Primex), and alkali refined soybean oil, were incorporated into control diets. The objective was to determine the subacute toxicity in rats of a low and a high potency tocopherol concentrate at high dietary levels.

Experimental

The fats and tocopherol concentrates evaluated in this subacute toxicity feeding trial are described in detail as follows:

1. Lard, prime, steam rendered.
2. Hydrogenated vegetable shortening (Primex).
3. Alkali refined soybean oil, hydrogenated (W-604-6).
4. Alpha tocopherol concentrate before distillation (13.7% tocopherols).
5. Alpha tocopherol concentrate - (25.5% tocopherols).

Each of these fats was added to a stock diet at a 12% level calculated to furnish 30% of the calories. Dietary levels of tocopherols were 1.65% from the low potency concentrate and 3.06% from the high potency concentrate.

The other ingredients of the diet were: 30% wheat, 20% yellow corn meal, 5% crude casein, 21% skim milk powder, 7% linseed oil meal, 2% alfalfa leaf meal, 3% bone meal, 2% yeast, 1% liver, 1% NaCl, and 0.5% $CaCO_3$.

Five groups of 10 male rats each were maintained on the respective diets for 98 days. The animals were weighed twice a week for the first four weeks and once a week thereafter. Changes in external appearance were looked for periodically. Food consumption was recorded during the second and final weeks of the experiment.

Results

The growth responses obtained are recorded in Table I and in Figure 1. All rats grew normally and no statistically significant differences were observed between group responses. Data on food consumption and efficiency of food utilization are summarized in Table II. There were no differences in food consumption or efficiency of food utilization between the experimental and control groups. No adverse effects of the experimental diets were observed during the experiment.

Discussion

The results of this experiment indicated that both the low potency and high potency alpha tocopherol concentrates at a 12% dietary level supported the growth of rats and permitted food utilization equivalent to that of the edible control fats. The results further indicate that dietary levels of tocopherols as high as 3.06% are innocuous when fed to rats for 98 days. Based on an average food intake of 15 g/day, the daily

intake per rat was 460 mg of tocopherols in addition to the tocopherol supplied by the basal diet. Thus, in the rat, tocopherols fed at more than 500 times the estimated daily requirement for vitamin E were without adverse effect.

Conclusions

It is concluded that alpha tocopherol concentrates derived from soybean oil fed at a dietary level of 12% (up to 3.06% tocopherols) supported normal growth and food utilization in rats.

Co. ny Code [redacted]
Sub. No. 0227 - Tocopherols Concentrate, Mixed
Box 55 (Question 7b)

Table I

Growth Responses of Rats Fed 12% Dietary Levels of
Alpha Tocopherol Concentrates and Three Edible Control Fats

Group	Fat	Average Weights (g) at various day intervals								Average Total Gain in Wt. ± S.E.
		0	14	28	42	56	70	84	98	
1	Lard	45.5	119.5	189.0	246.2	291.1	317.9	344.0	368.0	323.0 ± 17.4
2	Hydrogenated Vegetable Shortening (Primex)	45.0	124.8	196.0	254.5	287.4	311.0	329.0	346.7	301.7 ± 12.2
3	Alkali refined soybean oil (W-604-6)	47.7	126.2	203.7	263.8	294.3	321.2	346.0	360.1	312.4 ± 11.4
4	Alpha Tocopherol Concentrate (13.7% Tocopherol)	44.6	125.0	197.6	259.4	301.7	327.9	351.4	373.6	329.0 ± 12.8
5	Alpha Tocopherol Concentrate (25.5% Tocopherols)	45.9	124.3	193.0	251.1	287.9	302.6	328.4	345.7	299.8 ± 7.4

C ıny Code [redacted]
Sub. No. 0227 - Tocopherols Concentrate, Mixed
Box 55 (Question 7b)

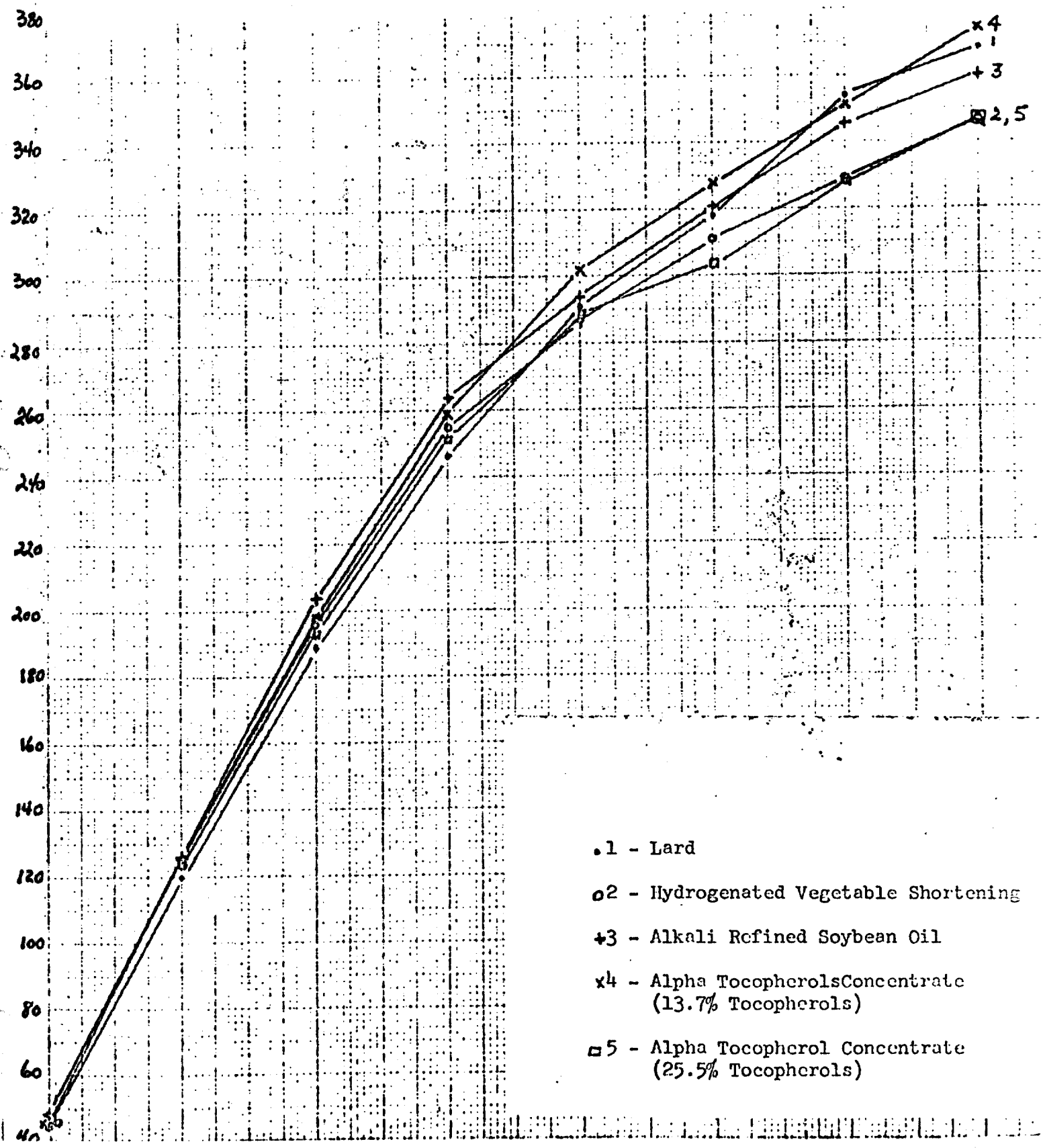
Table II

Food Consumption and Utilization of Rats Fed 12% Dietary Levels of Alpha Tocopherol Concentrates and Three Edible Control Fats

Group	Fat	14-21 days			91-98 days
		Total Food Intake	Total Gain in Wt.	Gain Wt. (g) per g Food Intake	Total Food Intake
1	Lard	87.9	32.1	0.37	102.2
2	Hydrogenated Vegetable Shortening (Primex)	101.9	37.5	0.37	111.4
3	Alkali Refined Soybean Oil (W-604-6)	108.1	36.4	0.34	120.7
4	Alpha Tocopherol Concentrate (13.7% Tocopherols)	103.6	33.8	0.33	125.1
5	Alpha Tocopherol Concentrate (25.5% Tocopherols)	100.2	36.4	0.36	125.3

Figure 1

Food Consumption and Utilization of Rats Fed 12% Dietary Levels of Alpha Tocopherol Concentrates and Three Edible Control Fats



By : Dr. H. Hummler, Dr. E. Theiss

Department : VI/Med.

Date : June 24, 1966

Subject : Tolerance Trial by the Oral Route for 12 Weeks in Rats

Theme : 4603

Preparation : Ro 1-4557 = alpha-tocopheramine

compared with

Ro 1-4147 = D-alpha-tocopherol

Ro 1-4213 = DL-alpha-tocopherol

Summary

Alpha-tocopheramine is an analog of the alpha-tocopherols. The substance should soon be tested in a field test as an anti-oxidant for food oils. The following report informs of the results of a 12-week tolerance study in rats, in which alpha-tocopheramine was tested for toxicity in comparison with d-alpha-tocopherol and dl-alpha-tocopherol. Outside of hyperplstic changes in the thyroids at 500-1000x (200 mg/kg) the proposed human dose, no evidence of toxic effects of the preparation appeared. In contrast to the proposed addition of 100 mg/kg of food fat there existed no doubt.

Alpha-tocopheramine should be tested for its qualifications as an antioxidant for food oils. In the following report will be reported a 12-week tolerance study on rats in which alpha-tocopheramine was examined in comparison with d-alpha-tocopherol and dl-alpha-tocopherol.

Methods

Groups of 5 male and 5 female, growing Fullinsdorfer albino rats of random rearing with a starting weight of 80-100 g received the preparation oraally (administered by throat probe). The substances were suspended in aqueous emulsions (cremophor) and administered in daily doses of 200, 700 and 2000 mg/kg during three months on the 5 workdays of the week.

A homogeneous control group received daily per os an analogous quantity of the aqueous emulsion without the additional preparations.

The diet consisted of "NAFAG" and tap water ad libitum.

Investigation

1. Body weight and general condition was checked every work day.

2. Blood tests: At the end of 6 weeks and at the conclusion of the test, hemoglobin level, hematocrit value, leukocyte- , erythrocyte- , reticulocyte- , and thrombocyte-counts were determined, as well as a prudent determination of the differential white count.

3. Urinalysis: After 6 weeks and at the conclusion of the test, with the 6-hour-urine of the control animals and of the animals receiving the highest dodes (2000 mg/kg) the following values were determined or tests executed: volume, pH-value, albumin, lactic acid dehydrogenase (LDH), specific weight, glucose, bilirubin, hemoglobin, acetone and acetic acid, phenylketone, sediment.

4. Function tests and other clinical, chemical tests: After 6 weeks and at the end of the experiment the following tests were carried out on the contols and the animals of the 2000 mg/kg groups: determination of glutamate-pyruvate-transaminase (GPT), glutamate-oxalate-transaminase (GOT), alkaline phosphatase, leucine-aminopeptidase (LAP), bilirubin and urea in the plasma, measure of the bromsulphalein (BSP)- plasma platlets 15 or 30 minutes after intravenous application of BSP (liver function test), renal phenol red excretion and diuresis test (kidney function test).

5. Histological examinations: The heart, lungs, liver, kidneys adrenals, spleen, stomach, intestines, gonads (and uterus of the females), thyroid and bone marrow of all animals were microscopically examined.

Results

1. Body weight and general health: Outside of the mild diarrhea during the first two weeks in some of the rats of the highest dose group, no significant influence of the preparations on behavior or general health could be ascertained. No difference in growth existed between the control and the treated groups (see figures in appendix). One control rat died of "lung probing" and another from an overdose of ether at the blood test. A rat of the highest dose group of the alpha-tocopheramine animals died shortly before the end of the study because of "lung probing". Ether overdosing and "lung probing" can also be assumed to be the cause of 2 cases of

death of the d-alpha-tocopherol-animals and the dl-alpha-tocopherol-animals. Otherwise no spontaneous deaths.

2. Blood tests: Either after 6 weeks or at the end of the experiment the differences between treated and untreated animals were diagnosed (see Tables 1-3 for average values, individual values in the appendix).

3. Urinalysis: Tests carried out after 6 weeks and at the end of the study produced no values or findings for the treated animals which differed from the norm (see Tables 4-11 for individual and average values).

4. Function tests and other clinical, chemical examinations: The tests carried out after 6 weeks and at the end of the study disclosed no differences between the treated and untreated animals (see Tables 12-19 for individual and average values).

5. Histological examinations: The histological findings of the rats treated with alpha-tocopheramine, d-alpha-tocopherol and dl-alpha-tocopherol were analogous to those of the control animals. Exceptions are mentioned in the following, summarized, histological description.

Heart: Muscle fibers were clearly streaked throughout, either vacuolized or degenerated, not pigmented. Nuclei very regular, sharply contoured, chromatin-rich. Interstitium fine-grained. Endo- and epicardium smooth. No inflammatory or degenerative processes.

Lungs: Individual lymph cell aggregations, isolated small, fresh intra-alveolar hemorrhages, isolated small bronchopneumonia centers in all groups. In the others regular alveolar cells, fragile septum and unchanged bronchioli.

Liver: Lobule structure regular. In individual animals of all groups, minimal to slight, "fine-dropped", partly lobule-periphery, partly lobule-center fatty degeneration. Pigment artifacts often did not allow themselves to be excreted. Likewise, in all groups, animals with small, chronically inflamed infiltrations in parenchyma and peri-portal areas. Sharply contoured, regular nuclei with small nucleoli. No necrosis.

Kidneys: Delicate, regularly structured glomeruli, intact tubule epithelium. In all groups, individual, small, interstitial fat-cell infiltrates. Blood vessels normal.

Adrenals: Typically stratified, with well differentiated cortical zones and equal, normal lipoid content. Nuclei regular and chromatin-rich.

Spleen: Capsule and trabecula delicate. Red pulp rich in blood. Without abnormal pigmentation. Follocles regular. In the nuclei centers no increase of nuclei fragments.

Stomach and intestines: Mucous membranes intact; no ulcerations, no inflamed infiltration. Muccular and visceral peritoneum without peculiarities.

Testes: Active nuclei in test- and contol-animals: all stages of speriogenesis and spermiohistogenesis evident.

Ovaries: Normally structured stroma. In addition to numerous corpora lutea, follicles of different stages of maturity.

Uterus: Myometrium delicate, of usual thickness. Endometrium corresponds to the various cycle phases.

Bone marrow: Rich in cells, no deviation in the distribution picture of the different cell forms, only slight, graduated fat-tissue streaks.

Thyroid: Alpha-tocopheramine: In the highest dose group (2000 mg/kg) in part, distinct follicular hyperplasia. Small follicles with high, cubic or cylindrical epithelium and little colloid. Also in the middle (700 mg/kg) and only very slightly in the lowest group (200 mg/kg), mild hyperplastic changes were ascertained.

D-alpha-tocopherol and dl-alpha-tocopherol: Only in the highest dose group (2000 mg/kg) , in part, very mild hyperplastic changes in the thyroids, however with high colloid. The remaining dose groups showed no changes differing from the controls. The epithelial cells are regularly structured.

Discussion

Alpha-tocopheramine is an analog of the alpha-tocopherols. The hydroxyl group is replaced with an amino group. The substance has approximately the same efficacy as alpha-tocopherol. It should soon be tested in a field trial as an antioxidant for food oils.

In a twelve-week study, alpha-tocopheramine and the substances for comparison, d-alpha-tocopherol and dl-alpha-tocopherol, in oral daily doses of 200, 700, and 2000 mg/kg were well endured by rats. Extensive hematological, clinical-chemical, and histological investigations, urinalysis, as well as function tests were carried out. In the highest (2000 mg/kg) and middle (700 mg/kg) dose groups, the animals treated with alpha-tocopheramine exhibited, in comparison with the control animals, a follicular hyperplasia of the thyroids. Very mild hyperplastic changes of the thyroids were also found in the animals treated with the comparison substances d-alpha-tocopherol and dl-alpha-tocopherol. Similar observations were, as far as is known, previously only caused by alpha-tocopherol in rabbits[1]. The growth of the animals and the rest of the tests gave no evidence as to a toxic effect of the preparations.

Alpha-tocopherol caused, in doses of some 5-20 mg/kg after 1-3 months, hyperplastic changes in the thyroids of rabbits[2] (according to the preceeding investigation, also very mild changes in rats with substantially higher doses). Results similar to these caused by alpha-tocopherol in rabbits were obtained by application of alpha-tocopheramine to rats, however only with substantially higher doses of 700 or more mg/kg over more weeks. Only with 200 mg alpha-tocopheramine per kg are the existance of such changes significant. Principle toxicological differences do not exist in experimental tests on animals with alpha-tocopherol on the one hand and alpha-tocopheramine on the other.

A true vitamin E-hypervitaminosis of men is unknown. In the recommendations of the FAO/WHO[3] a daily consumption of tocopherol by men of up to 1 mg/kg body weight was unconditionally accepted. At the provided dose of 100 mg alpha-tocopheramine per kg of fat and a daily consumption of 50-100 g of food oil/day, the men received 0.1-0.2 mg tocopheramine per kg body weight. This dose amounts to 10-20% of the quantity of alpha-tocopherol accepted by the FAO/WHO and corresponds to only some 1-2% of the lowest dose group of alpha-tocopheramine (200 mg/kg), with which, in animal tests, hardly any hyperplastic changes of the thyroid could be observed. For reasons of comparison, it was pointed out that the men received some 0.5 mg/kg alpha-tocopherol with 100 g of fish liver. Therefore, in comparison with the administration of 100 mg of alpha-tocopherol/kg food fat, no consideration exists.

Summary

Alpha-tocopheramine is an analog of the alpha-tocopherols. The substance should soon be tested in a field test as an antioxidant for food oils. The following report informs of the results of a 12-week tolerance study in rats, in which alpha-tocopheramine was tested for toxicity in comparison with d-alpha-tocopherol and dl-alpha-tocopherol. Outside of hyperplastic changes in the thyroids at 500-1000x (200 mg/kg) the proposed human dose, no evidence of toxic effects of the preparation appeared. In contrast to the proposed addition of 100 mg/kg of food fat there existed no doubt.

1. Studer A., Zbinden G. and Uehlinger.: The Pathology of Avitaminosis and Hypervitaminosis. Handbuch der allg. Pathologie, Bd. 11, 1. Teil, Springer Verlag Berlin, Gottingen, Heidelberg 1962.

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Date September 23, 1970

Report from W. Pool and D. Hane

Research Department Experimental Pathology and Toxicology to Management

Acute Toxicity and Dog Tolerance Testing of Vitamin E Injectable

Acute Toxicity

Species	Route	LD_{50} mg/kg	LD_{50} ml/kg
Mice	i.m.	> 250	> 5
Rats	i.m.	> 250	> 5
Rabbits	i.m.	> 50	> 1

Dog Tolerance

A male dog tolerated intramuscular doses of 2.5, 5.0 and 10 mg/kg (0.2 ml/kg) without symptoms. Hematology and clinical chemistries were normal.

Preparation

1. Vitamin E C-80639 (C-80649)

Ingredients	mg/cc
dl-Alpha Tocopheryl Acetate	*55.0 mg
Disodium Edetate	0.1 mg
Emulphor EL-620 stripped	40.0 mg
Glycerin	50.0 mg
Sodium Acetate - 3 H_2O	0.4 mg
Acetic Acid Glacial	0.1 mg
Thimerosal N F	0.1 mg
Sodium Hydroxide q.s. to pH 6.3	
Water for Injection q.s.	1.0 cc

* 10% excess included

Procedures

Acute Toxicity (Mice)

CF-1S mice of both sexes weighing 17-25 grams were used in this experiment. The preparation was administered intramuscularly. Ten mice were used per dose level and were observed five days for mortality.

Acute Toxicity (Rats)

Wistar strain rats of both sexes weighing 175-240 grams were used in this experiment. The preparation was administered intramuscularly. Ten animals were used per dose level and were observed five days for mortality.

Acute Toxicity (Rabbits)

Albino rabbits of both sexes weighing 2.28-2.85 kg were used in this experiment. The preparation was administered intramuscularly. Three rabbits were used per dose level and were observed five days for mortality.

Dog Tolerance Testing

A male dog weighing 12.3 kg received single intramuscular daily doses of the compound. The dose was doubled each successive working day and doses of 2.5, 5.0 and 10 mg/kg were administered. Serum glucose and cholesterol determinations were made on control samples, during dosing (1 hour after dose) and five days after final dosing. Blood counts, hemoglobins, hematocrits, BUN's and blood enzymes were performed before treatment, one hour after final dosing, and five days after final dosing.

W. Pool

D. Hane

WP:DH:amn

Table 1

Acute Toxicity

Vitamin E

Species	Route	Dose mg/kg	# Dead/ # Dosed	LD_{50} mg/kg	LD_{50} ml/kg	Symptoms
Mice	i.m.	250	0/10	> 250	> 5	No symptoms noted.
Rats	i.m.	250	0/10	> 250	> 5	No symptoms noted.
Rabbits	i.m.	50	0/3	> 50	> 1	No symptoms noted.

Table 2

Dog Tolerance Testing
Daily Observations

Vitamin E

Date	Dose (mg/kg, i.m.)	Dog #373 Male, 12.3 kg
8/3/70	2.5	No symptoms. Ate all food.
8/4/70	5.0	No symptoms. Ate all food.
8/5/70	10.0	No symptoms. Ate all food.
8/6/70	No Drug	No symptoms. Ate all food.
8/7/70	No Drug	No symptoms. Ate all food.
8/8/70 8/9/70	Weekend	No observation. Ate all food.
8/10/70	No Drug	No symptoms. Ate all food. Survived 5-day period.

Table 3

Hematology

Preparation	Subject	Time	WBC cu.mm	Poly %	Lymph %	Mon %	Eos %	Baso %	Hematocrit %	Hemoglobin gms %
Vitamin E	373	Pre-drug	7000	78	18	3	-	1	47	15.3
		Post-drug	6800	80	18	2	-	-	45	13.5
		5-Days Post-drug	6300	76	22	2	-	-	46	15.7

Table 4

Function Tests

Preparation	Subject	Time	BUN (mg %)	SGO-T (S-F units/ml)	SGP-T (S-F units/ml)	Alkaline Phosphatase (Sigma u/ml)
Vitamin E	373	Pre-drug	12.0	17	10	0.9
		Post-drug	11.5	30	10	0.7
		5-Days Post-drug	11.5	17	19	0.9

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Table 5

Serum Glucose and Serum Cholesterol Levels

Vitamin E

Dose mg/kg	Dog #373 Serum Glucose mg%	Serum Cholesterol mg%
Pre-drug	108	171
2.5	---	---
5.0	105	167
10.0	94	143
5-Days Post-drug	128	143

INTRAOFFICE CORRESPONDENCE

TO Dr. R. Floody DATE January 19, 1971

FROM Dr. W. Pool and Miss D. Hane

SUBJECT Acute Toxicity of Vitamin E Injectable in Neonatal Rats

Summary

Intraperitoneal administration of 2000 mg/kg of Vitamin E injectable produced no mortality in neonatal rats (LD_{50} = > 2000 mg/kg).

Preparation

1. Vitamin E (50 mg/cc) C-80639

Procedure

Neonatal Wistar strain rats (not more than 24 hours old) of both sexes weighing 6-9 grams were used in this test. The Vitamin E preparation was administered intraperitoneally to ten animals per dose level. The neonatal rats were observed for five days and total mortality was reported.

Results

Acute Toxicity

Preparation	Dose mg/kg, i.p.	# Dead/ # Dosed	LD_{50} (mg/kg, i.p.)
Vitamin E	2000	0/10	> 2000

W. Pool

D. Hane

Alpha- and Gamma-Tocopherol in the Rat: in vitro and in vivo Tissue Uptake and Metabolism

I. R. PEAKE AND J. G. BIERI
Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT In order to compare tissue uptake and metabolism of α- and γ-tocopherol in the rat, vitamin E-deficient male rats were injected intraperitoneally with a 1:1 mixture of *dl*-[^{3}H]-α-tocopherol and *dl*-[^{14}C]-γ-tocopherol. Blood and tissues were removed from the rats at 10, 19 and 27 hours after injection. In vitro incubations of plasma from these rats with red cells from deficient rats were performed, and α- and γ-tocopherol exchange led to an equilibrium after 6 hours. At equilibrium the red cells showed a slightly greater affinity for γ-tocopherol than α-tocopherol. Similar results were obtained when deficient red cells were incubated with an aqueous dispersion of α- and γ-tocopherol. Tissues from the injected rats were analyzed both for total radioactivity and for tocopherols, and showed approximately equal uptake of the two tocopherols at 10 hours, but a more rapid loss of γ-tocopherol than α-tocopherol after this time. Plasma and red cells analyses confirmed the in vitro incubation results with γ-tocopherol being taken up to a greater extent than α-tocopherol. It was concluded that rat tissues take up α- and γ-tocopherol to approximately the same extent, and in red cells a slight preference for γ-tocopherol exists. Tissues, however, showed a more rapid disappearance of γ-tocopherol. J. Nutr. *101:* 1615–1622, 1971.

INDEXING KEY WORDS vitamin E · α-tocopherol · γ-tocopherol · tocopherol metabolism

Of the eight natural members of the vitamin E group of compounds, α-tocopherol has the highest biopotency in alleviating the many and varied vitamin E deficiency symptoms seen in animals. Bunyan et al. (1) have tabulated available data to emphasize this point. Most of the work concerning the biochemistry of vitamin E has been α-tocopherol orientated; but of the other tocopherols, γ-tocopherol is extremely widespread in nature and in many non-green plant tissues and oils is present to a greater extent than α-tocopherol.

The reason for the low biopotency of γ-tocopherol is obscure. Several workers have related it to inefficient absorption of this tocopherol by the intestine (2, 3) since in vitro experiments involving the restoration of respiration by liver slices from vitamin E-deficient rats, or the prevention of dialuric acid-produced hemolysis of red cells have generally shown a greater γ-tocopherol biopotency than seen in experiments where intestinal absorption of the tocopherol was involved (2, 4). Selective uptake of the different tocopherols by body tissues has also been proposed (5). However, Gloor et al. (6) concluded that α- and γ-tocopherol are both absorbed to the same extent, but that γ-tocopherol is eliminated from the body in a much shorter time, thus resulting in a rapid decrease in tissue levels of this tocopherol.

This report is concerned with the results of experiments in which the tissue uptake, metabolism and elimination of α- and γ-tocopherol were compared in the rat. The availability of *dl*-[^{3}H]-α-tocopherol and *dl*-[^{14}C]-γ-tocopherol facilitated the easy identification of both parent tocopherols and their metabolic products when used jointly in the same experiment.

Received for publication June 10, 1971.

MATERIALS AND METHODS

The two labeled compounds *dl*-[5-methyl-^{3}H]-α-tocopherol [1] and *dl*-[3,4-^{14}C]-γ-tocopherol [2] were diluted with purified unlabeled *dl*-α-tocopherol [3] and *dl*-γ-tocopherol,[4] respectively, before use to give known specific activities (400 cpm/μg). The radioactive label was thus used as a rapid method of estimation, results being reported as micrograms of tocopherol. These stock tocopherols were analyzed prior to each experiment and only used when radioactive purity greater than 90% could be demonstrated after thin-layer chromatography.

Male, weanling rats of the Sprague-Dawley strain were caged individually and fed a vitamin E-deficient diet [5] for several months. In the experiments to be described, 400-g rats were injected intraperitoneally with emulsions containing the two tocopherols, and fasted for the times indicated until killed. Blood was then taken from the abdominal aorta with a heparinized syringe and tissues were removed and stored at −20° until analyzed. The injected emulsions were prepared as follows: 2.5 mg of each tocopherol were dissolved in 0.1 ml of ethanol. Tween 80 (0.05 ml) was added and the total was made to 1.5 ml with 0.85% saline, the mixture being continuously stirred. The whole of the resulting emulsion was injected.

Red cells were obtained by centrifuging whole fresh blood at 1000 × *g* for 15 minutes at 4°, removing the plasma and washing the cells once in 0.85% saline. In vitro incubations of red cells with plasma or synthetic emulsions were performed on a Dubnoff shaking incubator at 37° in 25-ml conical flasks. Sampling of incubations was achieved by removing 1 ml of incubation mixture and centrifuging down the red cells (see above), which were then washed with saline. This saline wash was added to the plasma or emulsion obtained from the first centrifugation, and the combined sample was extracted in the same way as were the red cells. This involved the addition of methanol (3 volumes) cooled to −20° and extraction of the resulting mixture three times with redistilled hexane. After evaporation of the hexane under nitrogen, samples were counted for radioactivity on a scintillation counter,[6] liquifluor [7] having been added. [^{14}C] and [^{3}H] activity in the same sample were separated using the discriminate ratio method of Okita et al. (8). Thus the amount of each tocopherol present in red cells and plasma (or emulsion) from 1 ml of incubation mixture was found, giving the distribution of tocopherols between these two phases at any one time. This method of expressing results, rather than per milliliter of cells or plasma, gives a clear picture of the exchanges occurring in these experiments, and allows for results to be expressed as percentage distribution of the total tocopherol present.

Tissue samples in duplicate were saponified and extracted using the method of Bieri (9). Before analysis, 15 μg each of α-tocopherol, γ-tocopherol, α-tocopherylquinone, γ-tocopherylquinone and α-tocopherol dimer [8] were added to the tissue in the extraction tube. Upon obtaining the hexane tissue extract, an aliquot was counted to give total levels of [^{14}C] and [^{3}H] present. The remaining extract was evaporated to dryness and the lipid thin-layer chromatographed as a short line on silica gel G [9] plates. Developing solvent was 20% diethyl ether in hexane, and after development, each plate was sprayed with a solution containing one part liquifluor (previously diluted for use in scintillation counting) and three parts ethanol. The bands corresponding to α-tocopherol, γ-tocopherol, α-tocopherylquinone, γ-tocopherylquinone and tocopherol dimers (as marked by α-tocopherol dimer) were visualized under ultraviolet light and the corresponding areas of silica, plus the origin, scraped directly into counting vials. After the addition of liquifluor, they were counted for

[1] 2.0 mCi/μmole: Amersham Searle Corp., Arlington Heights, Ill. All of the compounds used in this study and identified as *dl*-tocopherols are designated *all-rac*-tocopherols according to the tentative rules of the IUNS Committee on Nomenclature, Nutr. Abstr. Rev. 40: 395, 1970.

[2] 1.87 μCi/μmole: Gift from Dr. O. Wiss, Hoffmann-LaRoche, Inc., Basle, Switzerland.

[3] General Biochemicals, Chagrin Falls, Ohio.

[4] Hoffmann-LaRoche, Inc., Nutley, N. J.

[5] Bieri and Prival (7). Diet composition (in %): vitamin-free casein, 22; salt mix, 6; stripped lard, 5; vitamin mix in sucrose, 2; sucrose to 100%.

[6] Packard Model 3007, Packard Instrument Co., Inc., Downers Grove, Ill.

[7] New England Nuclear Corp., Boston, Mass.

[8] Kindly prepared by potassium ferricyanide oxidation of α-tocopherol by Dr. L. A. Cohen, Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

[9] E. Merck, Brinkman Instruments, Inc., Westbury, N. Y.

radioactivity as usual. Preliminary experiments had shown this counting method to be equally as efficient as methods involving extraction of material from the silica before counting.

RESULTS

In vitro experiments. Figure 1 shows graphically the establishment of equilibrium conditions when vitamin E-deficient red blood cells were incubated with plasma containing approximately equal amounts of *dl*-α-tocopherol and *dl*-γ-tocopherol. The curves obtained are very similar to those reported by Silber et al. (10) and Poukka and Bieri (11) for *d*-α-tocopherol, with equilibrium being reached in 6 to 8 hours. Both α- and γ-tocopherol appear to equilibrate in the same way, with perhaps a greater uptake of the latter by the red cells. Data from this and two subsequent experiments are given in table 1. In these experiments, plasma was obtained from rats killed at varying times after injection in order to give plasmas containing differing proportions of α- and γ-tocopherol, i.e., for the three incubations, α-tocopherol/γ-tocopherol ratios of 1.01:1, 1.42:1 and 1.96:1 were obtained. At equilibrium in these incubations, all plasmas had α-tocopherol/γ-tocopherol ratios higher than that of the initial plasma, while the ratios recorded in red cells were lower. Since no tocopherol destruction could be demonstrated, it was concluded that under these conditions, at equilibrium, a greater percentage of γ-tocopherol was present in the red cells than of α-tocopherol. Percentage distribution figures, given at the bottom of table 1, show that in these three incubations an average of 29% of the α-tocopherol was present in the red cells at equilibrium, while 35% of the γ-tocopherol was found.

Experiments in which red cells from a rat injected with α- and γ-tocopherol, were incubated in plasma from a deficient rat were also performed (data are not reported). Results, as expected, showed similar distribution patterns at equilibrium for both α- and γ-tocopherol, with plasma

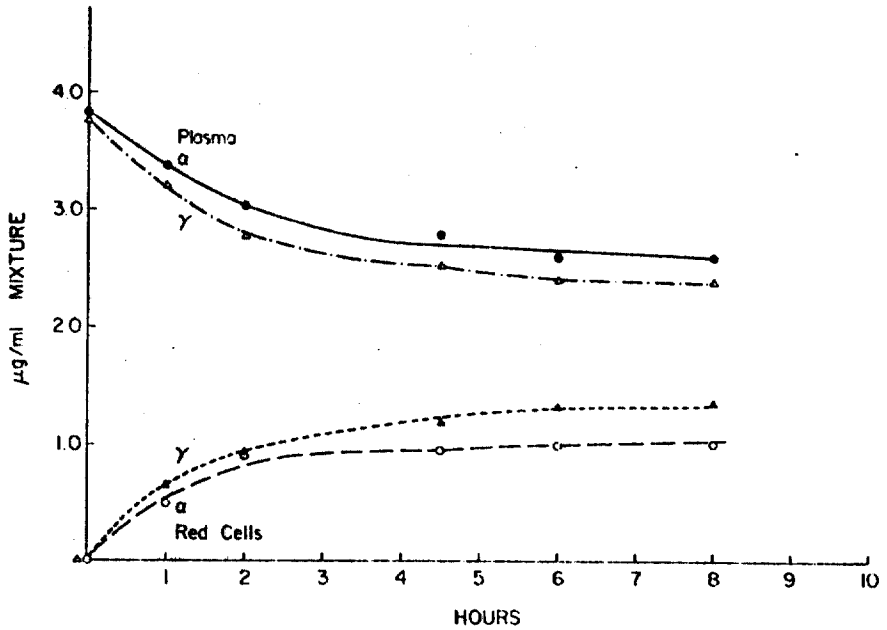

Fig. 1 Representative example of the incubation of vitamin E-deficient red cells with plasma containing *dl*-α-tocopherol (αT) and *dl*-γ-tocopherol (γT). Five milliliters of red cells were incubated with 5 ml of plasma and 1-ml aliquots were removed for analyzing at the times shown. Values are expressed as micrograms of tocopherol present in the red cells or plasma obtained from 1 ml of incubation blood. The incubation was performed at 37° and 1% glucose was added to the mixture. ●——● = αT plasma; Δ— · —Δ = γT plasma; ○- - - -○ = αT red blood cells; ▲ · · · · ▲ = γT red blood cells.

TABLE 1

In vitro incubation of vitamin E-deficient red blood cells (RBC) with plasma containing a mixture of del-α-tocopherol (αT) and dl-γ-tocopherol (γT)[1]

	Incubation 1					Incubation 2					Incubation 3				
	αT		γT		$\frac{\alpha T}{\gamma T}$	αT		γT		$\frac{\alpha T}{\gamma T}$	αT		γT		$\frac{\alpha T}{\gamma T}$
	μg	%	μg	%		μg	%	μg	%		μg	%	μg	%	
Initial plasma	3.82	100	3.77	100	1.01	3.46	100	2.44	100	1.42	2.29	100	1.17	100	1.96
Initial RBC	0	0	0	0		0	0	0	0		0	0	0	0	
Equilibrium plasma	2.61	70.4	2.39	63.4	1.09	2.48	71.9	1.63	66.8	1.64	1.57	70.4	0.73	64.0	2.38
Equilibrium RBC	1.10	29.6	1.38	36.6	0.80	0.97	28.1	0.73	33.2	1.07	0.66	29.6	0.41	36.0	1.78

[1] In each incubation, red cells from a deficient rat were mixed with plasma from a rat injected intraperitoneally with an emulsion containing the two tocopherols (described in text). Total volume of each incubation was 10 ml and hematocrits for incubations 1, 2 and 3 were 50%, 44% and 41%, respectively. The incubations were performed at 37° and equilibrium conditions obtained after 6 to 8 hours. Values are micrograms of tocopherol present in the plasma or red cells obtained from 1 ml of incubated blood.

containing proportionally slightly more α-tocopherol than γ-tocopherol.

In order to examine the exchange between plasma and red cells further, vitamin E-deficient red cells were incubated with an aqueous tocopherol dispersion instead of plasma. It was hoped in this way to remove any selective effect that plasma lipoprotein might have had on the red cell uptake. The clear tocopherol dispersions contained 0.025% Tween 60, 0.2% ethanol and 1.0% glucose, all in 0.85% saline solution. The tocopherols at concentrations similar to those used in the plasma incubations were within the normal rat plasma ranges of 500 to 1000 μg/100 ml plasma. Figure 2 shows the progress of such an incubation. Equilibrium was again reached in approximately 6 hours, and at this stage the red cells again contained proportionally more γ-tocopherol than α-tocopherol. This figure also shows the more rapid uptake of γ-tocopherol than α-tocopherol by red cells prior to equilibrium.

The data from three incubations using tocopherol dispersions are given in table 2. Initial ratios (α-tocopherol/γ-tocopherol) of 0.46:1, 0.94:1 and 1.80:1 were used and in all cases, at equilibrium, the red cell ratio was lower than the initial dispersion ratio. Percentage distribution figures of the tocopherols in the two phases emphasize this.

In vivo experiments. Plasma and red blood cell concentrations of α- and γ-tocopherol from rats killed at different times after an intraperitoneal injection of the two tocopherols (2.5 mg each) are given in table 3. Considerable variation occurred in the levels from rats killed at the same time but, generally, proportions of α-tocopherol/γ-tocopherol were higher in the plasma than in the red cells. Also, with increase in time, the ratio of α-tocopherol/γ-tocopherol tended to increase, both in the plasma and, since red cells are in equilibrium with the plasma, also in the red cells. (Rat number 6 appears to be an anomaly. The reason for this is not clear and is probably indicative of the unusal situation produced in rat blood by the administration of a large dose of tocopherol intraperitoneally combined with continuous exchange by tissues, and continuous metabolism and elimination from the body.)

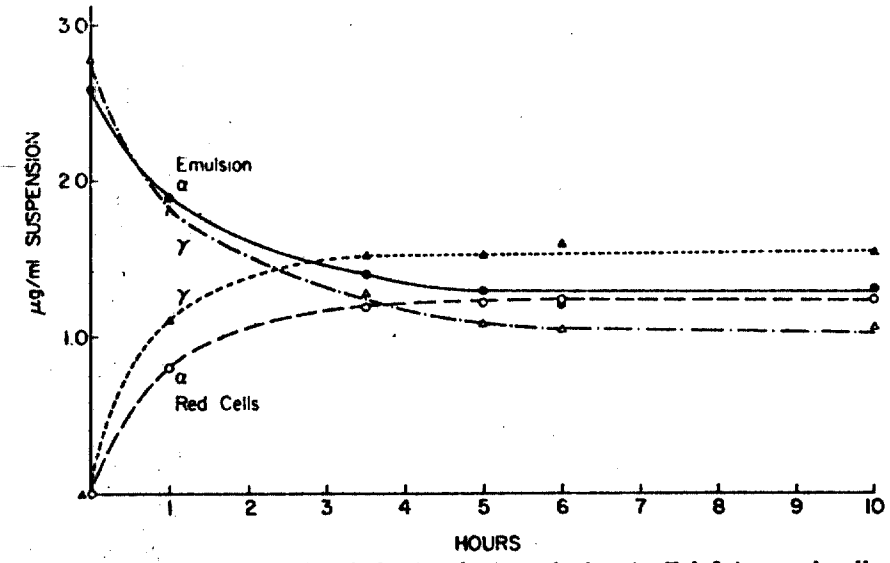

Fig. 2 Representative example of the incubation of vitamin E-deficient red cells with an aqueous dispersion containing *dl*-α-tocopherol (αT) and *dl*-γ-tocopherol (γT). Four milliliters of red cells were mixed with 6 ml of dispersion and 1-ml aliquots were removed for analysis at the times shown. Values are expressed as micrograms tocopherol present in the red cells or plasma obtained from 1 ml of incubation suspension. The incubation was performed at 37° and the emulsion contained 0.025% Tween 60, 0.2% ethanol, 1% glucose, all in 0.85% saline ●——● = αT dispersion; △—·—△ = γT dispersion; ○— —○ = αT red blood cells; ▲····▲ = γT red blood cells.

From each of the six rats in table 3, various tissues were removed and analyzed (table 4).[10] Highest concentrations occurred in the spleen and liver, with thigh muscle containing the lowest concentration. Although the levels in tissues from rats killed at the same time varied two- or threefold, ratios of α-tocopherol to γ-tocopherol were fairly constant. At 10 hours, all tissues showed a ratio of approximately 1.0 with perhaps spleen and liver possessing more γ-tocopherol than α-tocopherol. After 10 hours, the tissues can be roughly divided into three groups. Spleen and liver showed a drop in tocopherol concentrations, but with the ratio of α-tocopherol/γ-tocopherol remaining approximately constant. Heart and kidney showed an increase in ratio at 19 hours, due to increased levels of α-tocopherol, but little subsequent change was seen at 27 hours. Testes and muscle, however, showed a steady rise in ratio up to 27 hours, due to a combined increase in α-tocopherol level and a drop in γ-tocopherol.

Thin-layer chromatographic separation of α-tocopherol and γ-tocopherol in heart, kidney, testes and muscle extracts showed that 75% to 85% of radioactivity was present as unchanged tocopherol at all times. The remaining activity was not present in any one material, but spread through the remaining area of the chromatogram. However, in the case of spleen and liver, recovery of [^{14}C] activity as unchanged γ-tocopherol was low, especially in the 27-hour rats. Table 5 tabulates the percentage recovery of radioactivity from thin-layer chromatograms of samples of liver from rats killed at 19 and 27 hours. At 19 hours, 12.9% of the ^{14}C activity was located in the dimer region, while at 27 hours this had risen to 43.5%. Only a small increase was seen for ^{3}H activity. Further studies have located most of the [^{14}C] dimer activity in a single ultraviolet absorbing band (R_F of 0.94 in 10% ether in hexane) with a maximum absorption between 292 and

[10] Levels calculated from total [^{3}H] or [^{14}C] found in each hexane extract.

TABLE 2

In vitro incubation of vitamin E-deficient red blood cells (RBC) in an aqueous dispersion containing dl-α-tocopherol (αT) and dl-γ-tocopherol (γT)[1]

	Incubation 1					Incubation 2					Incubation 3				
	αT		γT		$\frac{\alpha T}{\gamma T}$	αT		γT		$\frac{\alpha T}{\gamma T}$	αT		γT		$\frac{\alpha T}{\gamma T}$
	μg	%	μg	%		μg	%	μg	%		μg	%	μg	%	
Initial dispersion	1.37	100	2.95	100	0.46	2.59	100	2.77	100	0.94	2.68	100	1.49	100	1.80
Initial RBC	0	0	0	0		0	0	0	0		0	0	0	0	
Equilibrium dispersion	0.81	60.4	1.43	50.0	0.57	1.21	49.4	1.05	39.8	1.15	1.60	59.7	0.75	50.3	2.13
Equilibrium RBC	0.53	39.6	1.43	50.0	0.37	1.24	50.6	1.59	60.2	0.78	1.08	40.3	0.74	49.7	1.46

[1] In each incubation, red cells from a deficient rat were mixed with an aqueous tocopherol dispersion to give a total volume of 10 ml. hematocrits for incubation 1, 2 and 3 being 50%, 40% and 50%, respectively. Dispersions were prepared in 0.85% saline and contained 0.025% Tween 60, 0.2% ethanol and 1% glucose, plus the tocopherols. Incubations were performed at 37° and equilibrium conditions were attained in 6 to 8 hours. Values are micrograms of tocopherol present in the dispersion or red cells obtained from 1 ml of incubated mixture.

295 nm, and mass spectra have revealed a large molecular ion at m/e = 830, indicating a dimer of γ-tocopherol. Studies on the nature of this dimer are continuing.

DISCUSSION

In vitro experiments in which vitamin E-deficient rat red cells were incubated with plasma or aqueous dispersions containing both α- and γ-tocopherol have shown that at equilibrium γ-tocopherol is present in the red cell to a slightly greater extent than α-tocopherol. They have also demonstrated that this phenomenon is a result of red cell activity, and not due to selective binding of tocopherols to lipoproteins in plasma, since aqueous tocopherol dispersions produced the same result. The opposite experiments in which red cells containing the two tocopherols were incubated with deficient plasma have confirmed this finding, as have in vivo measurements of tocopherol levels in the plasma and red cells of rats injected intraperitoneally with the two tocopherols. These latter measurements show a ratio of α-tocopherol/γ-tocopherol higher in the plasma than in the red cells (table 3). In considering the significance of these results, two facts must be borne in mind. First, in vitro studies of the prevention of dialuric acid-induced hemolysis in rat red cells by the addition of tocopherol have shown γ-tocopherol to be only two-thirds as effective as α-tocopherol (Bunyan et al. (4)). However, in terms of antioxidant activity, γ-tocopherol is generally considered to be more efficient than α-tocopherol in vitro. Hove and Hove (12) reported that γ-tocopherol is 6% less efficient in preventing peroxidation of ethyl oleate by aeration at 35°, but this difference cannot account for the observed biopotencies. Since these studies have shown at least an equal uptake of the two tocopherols by the red cells, the biopotency differences cannot be accounted for adequately in terms of uptake or antioxidant activity.

In considering the tissue analyses, uptake of the two tocopherols again appeared to be approximately equal, as seen from the 10-hour figures in table 4. However, γ-tocopherol disappeared from the tissues at a considerably faster rate than α-tocopherol. Even in the spleen and liver, where

TABLE 3

Plasma and red blood cell tocopherol levels at varying times after the intraperitoneal injection of a mixture of α-tocopherol (αT) and γ-tocopherol (γT) [1]

Rat no.	Time	Plasma			Red blood cell		
		αT	γT	$\frac{\alpha T}{\gamma T}$	αT	γT	$\frac{\alpha T}{\gamma T}$
	hr	*μg/ml*	*μg/ml*		*μg/ml*	*μg/ml*	
1	10	8.96	9.42	0.95	1.25	2.37	0.53
2	10	6.18	4.36	1.42	3.42	3.63	0.93
3	19	3.88	1.98	1.96	5.00	3.08	1.62
4	19	5.18	2.85	1.82	5.68	4.09	1.39
5	27	4.38	2.41	1.82	3.86	3.47	1.11
6	27	3.00	1.44	2.08	5.66	2.37	2.38

[1] Each rat was injected with 2.5 mg each of α-tocopherol and γ-tocopherol and killed after the indicated time. All levels are micrograms of tocopherol per milliliter of plasma or packed red cells.

TABLE 4

Tissue levels of α-tocopherol (αT) and γ-tocopherol (γT) from rats injected intraperitoneally with an emulsion of these two tocopherols [1]

		10 hours		19 hours		27 hours	
		μg/g	$\frac{\alpha T}{\gamma T}$	μg/g	$\frac{\alpha T}{\gamma T}$	μg/g	$\frac{\alpha T}{\gamma T}$
Spleen	αT	133, 269	0.72, 0.82	218, 341	1.12, 1.26	58, 141	0.93, 1.05
	γT	184, 303		194, 273		54, 152	
Liver	αT	42, 52	0.81, 0.89	29, 41	1.08, 1.26	16, 51	1.11, 1.29
	γT	47, 65		27, 31		12, 46	
Heart	αT	1.2, 2.5	0.89, 0.99	2.3, 2.7	1.39, 1.51	2.1, 2.5	1.20, 1.52
	γT	1.3, 2.4		1.6, 1.8		1.3, 2.1	
Kidney	αT	0.6, 1.3	0.89, 0.99	2.3, 2.5	1.65, 1.83	1.6, 2.4	1.42, 1.94
	γT	0.7, 1.3		1.3, 1.5		0.8, 1.7	
Testes	αT	0.4, 0.6	0.92, 1.02	1.0, 1.2	1.45, 1.47	0.8, 1.0	1.42, 2.00
	γT	0.4, 0.6		0.7, 0.8		0.5, 0.6	
Muscle (thigh)	αT	0.5, 0.6	0.89, 1.21	0.4, 0.5	1.40, 1.50	0.6, 0.8	2.00, 2.20
	γT	0.4, 0.6		0.3, 0.3		0.3, 0.4	

[1] Each rat was injected with 2.5 mg of each tocopherol. Values for two rats are given as μg/g fresh weight calculated from the total [^{3}H] (αT) or [^{14}C] (γT) activity in each analysis.

TABLE 5

Distribution of radioactivity from thin-layer chromatographic separation of liver lipid [1,2]

Fraction	R_F	[^{3}H] Activity (αT)		[^{14}C] Activity (γT)	
		19 Hours	27 Hours	19 Hours	27 Hours
		%	%	%	%
Dimer	0.87–1.0	2.0	8.7	12.9	43.5
Tocopherol	0.35–0.50	94.8	85.4	87.1	53.7
Quinone	0.10–0.15	2.2	4.0	—	0.9
Origin	0.00–0.10	1.0	1.9	—	1.9
Total		100	100	100	100

[1] Tissues were from rats described in table 4.
[2] Four fractions were removed from chromatograms developed in 20% diethyl ether in hexane. Each fraction was between the R_F values shown and negligible activity was found in the remainder of the chromatogram. Dimer activity corresponds to that material on the plate cochromatographing with α-tocopherol dimer and includes the solvent front. Values are percentage recovery of the total recovered activity from each chromatogram.

this was not apparent from initial radioactivity analyses, further fractionation revealed a much lower recovery of γ-tocopherol than α-tocopherol (see table 5). This loss of γ-tocopherol may be the reason for the low efficiency of γ-tocopherol when compared to α-tocopherol in the treatment of deficiency symptoms (e.g., muscular

dystrophy in the chick, $\alpha T = 100$, $\gamma T = 5$ as reported by Scott and Desai (3)), as was proposed by Gloor et al. (6). The high levels of both tocopherols found in spleen may be due to the reticuloendothelial function of this organ in removing foreign material from the body.

The presence of the unknown product of γ-tocopherol in spleen and liver may point to the way in which γ-tocopherol is removed from the tissues. The product was also detected in heart tissue at 27 hours after injection, but was at a level of less than 10% of the total [^{14}C] activity present. It is probably significant that it was detected only in the tissues which accumulated the highest concentration of tocopherols. The increase with time in the level of this γ-tocopherol metabolite, presumed to be a dimer, in liver and spleen would tend to discount the possibility that it was formed in the extraction of the tissues. Many tocopherol "metabolites" can be formed during extraction of tissue if care is not taken. The stability of α-tocopherol found here is in good agreement with the data of Plack and Bieri (13) who recovered 80% of radioactivity as unchanged α-tocopherol in the liver of rats 48 hours after intraperitoneal injection.

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Rat – natural tocopherols – absorption – small intestine

Absorption of Tocopherols by Small Intestinal Loops of the Rat *in vivo*

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Abstract: About 600–800 µg α, β, γ or δ-tocopherol in 0.2 ml glycerol trioleate were injected into small intestinal loops of hooded female rats and the disappearance of each was studied.

It was found that 32 % of the α-tocopherol, 18 % β-tocopherol, 30 % γ-tocopherol and 1.8 % δ-tocopherol disappeared over the 6 hour period.

It has been shown previously that the relative efficacy of absorption of the naturally occurring tocochromanols as judged by measuring tissue levels at timed intervals after oral dosing, is α > β > γ > δ-tocopherol and the relative extent was shown to vary with time [3, 7a, 7b]. A differential "barrier" or "barriers" must therefore exist between the intestinal lumen and the tissue cells. These "barriers" could occur at the intestinal mucosa, the blood vessel walls and/or at the tissue cell plasma membranes. Other physico/chemical factors such as micelle formation in the intestinal lumen and carriage in the blood and lymph may also effect the ultimate tissue levels of tocochromanol. This work attempts to establish the extent of differential uptake at the intestinal surface.

It has been shown that the order of disappearance from rat intestinal loops was α > β + γ > δ when they were given in a mixture [5]. However, it was found that synergistic absorption occurred when a mixture of d-α and l-α-tocopherols was given to rats; 50 % more was absorbed when they were administered together than when they were given singly [11]. It was decided therefore, to study the disappearance of α, β, γ and δ-tocopherols from rat intestinal loops *in vivo* when the tocopherols were administered singly.

Methods

Animals and diets

Female hooded rats weighing about 160–200 g were used throughout. They were maintained on a food cube diet (Spital No. 4, Silcock & Lever Feeds Ltd., Bromborough, Cheshire).

Chemicals

Natural tocopherols (Distillation Products Industries, Rochester, New York, U. S. A.) were used throughout. They were purified by preparative thin layer chromatography before use.

The tocopherols were mixed with glycerol trioleate (B. D. H. Chemicals Ltd., Poole, England) as carrier. This was analysed and found to be free of tocopherols.

Preparation of loops and dosing of rats

Rats were starved overnight and intestinal loops were prepared the following day. The animals were anaesthetised by an intraperitoneal injection of 0.1 ml 0.6 % pentobarbitone sodium (Nembutal, Abbott Laboratories Ltd., Queenborough, England)/100 g body weight. A mid-line abdominal incision about 3" long was made and the stomach and duodenum were located. A ligature was placed around the duodenum ½ cm from the stomach and another loose thread ligature was placed about 1 cm from the stomach. The caecum was then located and a ligature placed just proximal to the ileo-caecal junction. A hypodermic needle was inserted between the two duodenal ligatures extending into the lumen beyond the loose thread ligature which was then pulled tight around the needle and the dose was injected slowly. As the needle was removed the ligature was pulled tight and tied. The rats were not allowed to recover during the experiment.

Each rat received a single dose of about 600–800 µg of α, β, γ or δ-tocopherol in 0.2 ml glycerol trioleate. The rats were killed 0, ½, 1, 2, 3, 4, 5, or 6 hours afterwards and the small intestine (inclusive of ligatures) and the livers were removed for tocopherol assay. Two rats were killed at each zero time and the average taken as the amount of tocopherol given. This figure is considered as 100 % recovery, or 0 % disappearance, and the recoveries at other times were compared with it to ascertain the magnitude of disappearance from the loops. One rat was killed at other times.

Livers were also analysed in an attempt to correlate disappearance from the intestinal loops with deposition in the liver. All intestinal loops and livers were analysed for all four tocopherols to show the levels normally contained in these tissues, *e.* g. control levels of β, γ, and δ-tocopherols were determined in rats given α-tocopherol. This was necessary to evaluate the error which could result from indigenous tocopherols being estimated with the dose.

The method of tocopherol assay is reported elsewhere [7b].

Results

It can be seen from Fig. 1 that about 32 % of the αT, 18 % βT 30 % γT and 1.8 % of the δ-tocopherol disappeared over the 6 hour period. The points on the graph have a variance due to two main factors. There is variation between individual rats and between different groups of rats. This variation was minimised by using rats of the same strain, sex and approximate weight. The second factor is the variation due to tocopherols already present in the intestine. The averages of the tocopherols normally present are shown in Table 1. They are expressed as % of the dose given and this gives a measure of possible variation of each point on the graph due to this factor.

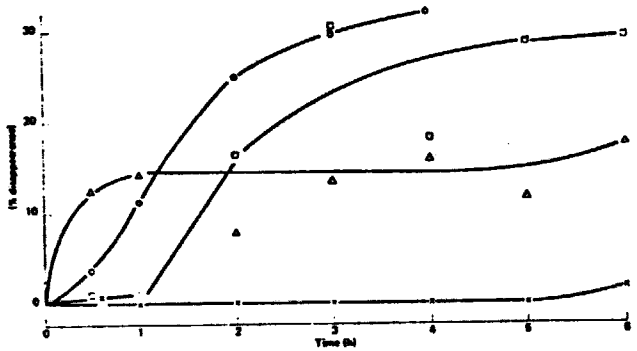

Fig. 1: Disappearance of tocopherols from rat small intestinal loops *in vivo* after intraduodenal administration of α, β, γ or δ-tocopherol in 0.2 ml glycerol trioleate.
Dose μg/rat αT 778, βT 700, γT 622, δT 770 μg.
○—○ αT, △—△ βT, □—□ γT, ×—× T

Tab. 1: Average tocopherol content of control rat small intestinal loops

Tocopherol	μg/loop	% dose
αT	34.7 ± 3.88 (27) *	4.46 ± 0.49
βT	14.2 ± 3.88 (24)	2.03 ± 0.55
γT	6.9 ± 1.64 (25)	1.1 ± 0.26
δT	0 (26)	0

* Mean ± S. E. M. (number of loops)

Discussion

The results indicate that the relative magnitude of disappearance from the intestinal loops was αT > γT > βT > δT. Although the difference between αT and γT may be within biological variation due to the tocopherol already present in the intestine, the difference between γT and βT would appear to be real. These findings are in contrast to the relative efficacies of absorption as judged by measuring tissue levels after oral administration whereby a greater percentage of the dose of βT than γT is deposited in the tissues [3, 7a, 7b].

Information about blood and lymph levels followings oral or intraduodenal administration of all four tocopherols would show whether the above results were due solely to differences in transport through the intestinal wall or to other factors as well. However, there does not appear to be any report on this. Measurement of blood levels in the pig after feeding a mixture of tocopherols showed that α-tocopherol was present in greater amounts than γ and δ-tocopherols [1]. Work with rats and rabbits has revealed the uptake of all four tocopherols into the blood stream but the β and γ-tocopherols were not separated prior to estimation [4, 5].

All the δ-tocopherol appeared to remain in the intestine except at 6 hours where 1.8 % of the dose had disappeared. The results obtained by Kaludin *et al.* showed that about 30 % of the δ-tocopherol had disappeared 4 hours after giving an intraduodenal dose of a mixture of α, β, γ and δ-tocopherols [5]. This reflects perhaps the occurrence of a synergistic effect [11]. These workers did not separate the β from the γ-tocopherol prior to estimation and therefore it is difficult to compare their results with those reported here. Loss of δ-tocopherol by absorption did in fact occur since trace amounts were found in the livers of rats killed 3 and 6 hours after dosing. The loss, however, was small and must have fallen within the experimental range of the isolation and determination of δ-tocopherol.

The loss of all tocopherols from the loops was less than 33 % which is in agreement with the known poor absorption of tocopherol in animals [2, 6, 8, 9, 10].

Measurement of liver levels did not reflect any pattern, since the amounts found there were usually in trace quantities, and are therefore not reported.

Acknowledgements. We are thankful to the Science Research Council for the support of CKP.

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THE INFLUENCE OF VITAMIN E ON BLOOD COAGULATION

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(From the Institute of Clinical Pediatrics of the University of Florence)

Florence section -- Meeting of December 6, 1949

Until a short time ago, the question of a possible influence of vitamin E on blood coagulation had never been taken into consideration. Several months ago, while I was performing some experiments on the behavior of glycemia, inorganic P, creatine and creatinine in children subjected to the oral administration of vitamin E (this research carried out at the Pediatric Clinic of Florence, in collaboration with F. Raggazini), I noticed that the blood withdrawn from the elbow vein 1 or 2, and even 3 hours after the oral administration of 100 mg vitamin E, was particularly thick and coagulated with particular speed, so that it often occluded the needle and made it impossible to withdraw the entire 10 cc of blood needed for the experiment.

This phenomenon, which repeated itself systematically upon every renewed attempt, incited me to test the rate of coagulation in some one of the subjects who had been administered the vitamin E. In this way I was able to observe that vitamin E had the power of reducing the time of coagulation.

From an examination of the literature, we see that vitamin E has interfered in the process of coagulation only in the work of L. Tropeano (1948), where the author reports that he cured a case of fibrinopenic syndrome by administering vitamin E in a daily dose of 25 mg for 25 days.

While my experiments were still in progress, a successive work by the same author revealed that he had been able to verify a marked decrease in the time of coagulation, whether *in vivo* or *in vitro*, after the administration of vitamin E.

Taking into account the fact that my experiments were joined nearly at the end, and that the mechanism of interpretation of such an action exercised by vitamin E still remained hidden, I decided to broaden the field of the experiment by researching as well the influence of this vitamin on the time of prothrombin, whether *in vivo* or *in vitro*.

I report here the results obtained in the experiments on the time of coagulation and the time of prothrombin in 23 subjects before, and 1, 2, and 3 hours after the oral administration of a single dose (100 mg) of vitamin E (Ephinal).

Besides this, in 13 subjects I tested the time of coagulation (in 4 the rate of prothrombin as well), collecting the blood (3 drops) from 11 children and 2 patients of the department, and

putting it immediately into a paraffinated microscopic slide in contact with a drop of a suspension obtained by mixing a tablet of Ephinal (50 mg) with 10 cc physiological solution at 37°.

The results of the research were as follows: in all 23 cases, the time of coagulation was much decreased as early as one hour after the administration of the vitamin. This reduction varied between 1/5 and 4/5 of the initial value.

In 17 cases, the greatest decrease was verified after the first hour, in 7 during the second, while in the third hour the time of coagulation approached the departure values, though always remaining beneath them, with the exception of 7 cases.

The time of prothrombin, tested with the classic method of Quicke in the same 23 subjects under the same experimental conditions, was reduced in 13 of the 23 cases, not considering the decreases to less than 4" which might be considered of little practical value. Particularly marked values (to 8") of such a reduction were verified in 9 cases.

In the tests conducted in vitro, the results were nearly identical: in all 13 cases the time of coagulation was notably reduced. In this last test, I wished to experiment with natural vitamin E as well, instead of α-tocopherol acetate (Ephinal), and for this end I employed vitamin E- Gelucap formed from V.C.A.: the decrease in the time of coagulation was even more marked, which fact invalidated our doubt that the excipient contained in the tablets of Ephynal had interfered in the phenomenon.

The results obtained, which reveal a new and interesting biological action of vitamin E, deserve further development in more experiments, especially such as would serve to clarify the point of attack of vitamin E in the delicate process of blood coagulation.

Of the hypotheses which we can pass in review here in a quick synthesis, we can reject the one which is based on the action of vitamin E as an element protecting the vasal endothelia, whose conditions of permeability it improves, as shown in tests in vitro: we can reject the hypothesis that such action is exercised directly on the plasmatic fibrinogen, because in experiments still in progress, this last demonstrates no alterations after the administration of the vitamin, and because in cases of hemophilia, in which the blood value of fibrinogen is normal, the time of coagulation was demonstrated as being influenced in the same sense by vitamin E; we can reject the hypothesis that vitamin E acts on the blood platelets, since neither their number nor their morphological appearance is altered, and because in Werlhoff's disease, where there is a constitutional thrombopenic anemia, vitamin E yielded, in our hands, no results.

It should be outlined that this vitamin interferes in the first phase of coagulation, that is, in that which allows the prothrombin in the presence of Ca-ions and thrombokinase, to transform into thrombin.

This of course only has the value of a hypothesis, a hypothesis which, however, already finds favorable support in the fact that vitamin E interferes in the exchange of cholesterol and the phosphoric esters, which, considering that thrombokinase is considered a phospholipide, is not without importance as a first clinical indication, constituted from the promising thera-

peutic results obtained by me in a case of hemophilia, a disease which, though there are still many uncertainties, would seem to have as a pathogenetic cause a deficiency in thrombokinase bound to particular conditions of resistance of the platelets.

However, it remains undisputed that vitamin E has revealed the capacity of notably accelerating blood coagulation.

Can the results of these experiments be interpreted as a first step toward a specific cure of hemophilia?

Further research still in progress will be able to offer clarification of this point.

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Institute of the Pediatrics Clinic of the University
of Florence
Director: Prof. C. Cocchi

A HISTOLOGICAL STUDY OF HYPERVITAMINOSIS E IN THE GUINEA PIG

Dr. Paolo Prosperi and Dr. Lorenzo Borselli

I. -- Introduction

It seemed to us to be of some interest to direct our attention to the problem of vitamin E dosage.

In these recent years, the study of this vitamin has been faced with obligation and seriousness in Italy as well, to the extent that it can now be said that we know much more about many aspects of this vitamin than was known in the past.

Extensive investigations have also shed light on the biochemistry and physiology of vitamin E.

Today we are nearly exactly aware of the blood level of vitamin E in man and in woman, as well as in some animals, under particular physiological conditions (pregnancy, labor); we are acquainted with the mechanism of absorption of vitamin E, and know which are the organs which effect its deposition, and which those which effect its elimination.

These documentations, often supported by specific experimental bases, have previously facilitated the clarification of the already known picture of avitaminosis E. On the other hand, some considerations emerging from such studies, especially those resulting from a knowledge of the mechanism of absorption and elimination of tocopherol, induced us to resume the study of hypervitaminosis E, to which we were also spurred by the divergent hypothesis existing in the clinical field concerning therapeutic dosages of this vitamin. In this last section, two periods can be distinguished in the evolution of our thoughts concerning it.

In a first period, which we can date as preceding the 2nd International Congress on vitamin E (New York 1949), the widespread opinion was the useful therapeutic dose must not much exceed the normal daily requirement of vitamin E.

This requirement was estimated as around 3-5 mg, which is less than the amount which is the vitamin E content of the ordinary American diet (24 mg per day according to P. Harris, 1950). In the same way, C. Hengel, at the said Congress, maintained that in order to be useful in any therapeutical application, vitamin E should be administered in a dose of 30 mg per day. In 1949, A.

Vogelsang, E. Shute and W. Shute began to administer doses of vitamin E amounting to around 150-200 mg per day, to patients of circulatory and cardiac ailments. The example of the Canadian authors found enthusiastic support among the following: J. Burgess (100-300 mg per day) in cases of ulcus cruris, H. Levy and E. Boas (200-800 mg per day) in cases of angina pectoris, J. Burgess again (100-600 mg) in cases of stasis ulcers, and A. Vogelsang (300 mg) in cases of diabetes, U. Butterini in cases of diabetes, P. Prosperi and A. Lottini, 1950 (100-300 mg per day) in cases of hemophilia, and after 1950, nearly all the authors who reported on the therapeutic use of the vitamin in various diseased condition employed higher doses.

Reports concerning the danger of elevated doses are very scarce. The Shutes, for example, initiate the cure cycle of cardiac diseases with doses of 900 mg per day, maintaining that this high dose is not contraindicated by the chronic rheumatic lesions of the heart and the intense hypertension. According to H. Levy and E. Boas, on the other hand, massive doses of vitamin E can sometimes provoke cephalea and vertigo, without its being possible to define the exact significance of this disadvantage.

For the purpose of preventing hemorrhaghic manifestations in the new-born, P. Prosperi and S. Dall'Orso (1953) administered vitamin E orally in a dose of 500 mg per day during the last days of pregnancy and verified no disturbances; rather, there was an of the advantageous effect of the vitamin on the duration of labor and the blood losses occuring during the same (data supported by the reduction of coagulation and Howell time in both the mother and the infant). A. Wagner (1951) and A. Biidendijn and F. Noordhoek (1951) also used high doses of vitamin E (300 mg per day) in the treatment of cutaneous ulcers, without noting any disadvantages. The clinic experimentation followed for seven years in the Florentine Pediatrics Clinic was also confirming concerning the absence of disadvantages derived from high doses of vitamin E. Since, however, there is no lack of experiments which speak of both clinical and experimental consequences of hyperdoses of vitamin E, we wanted to control this possibility experimentally.

We refer here to Reiss, who, in 1941, experimenting on female rats with high doses of synthetic vitamin E (orally and parenterally administered), produced menstrual irregularity, a numerical decrease of the yellow bodies, and total weight diminutio of the ovary. F. Bicknell (1947) also maintains that vitamin E in high doses can, both in the pregnant woman and in the rat, lead to a sudden insurgence of convulsions, and can unfavorably influenc the course of diseases of the nervous system, causing muscular lesions or provoking decalcification of the bones. The recent research of F. Bodenheuer and W. Lesch (1950), conducted on a small Levante field mouse subjected to administrations of 5 g of wheat germ oil and 50 mg pure alpha-tocopherol per day, revealed, in the female, hypertrophy of the interstitial tissue, suppression of the formation of primordial follicles, death of the follicle eggs in the process of maturation. According to the same authors, the administration of vitamin E for a long period leads to an intense intoinigation of the cells of the theca interna and the interstitial tissue, to a great increase in fibrocytes, the increase in thickness of the vessel walls, with restriction of light and

those obtained by the application of X-rays to the ovary, are completely absent under the same experimental conditions in the genital organs of the male rat.

Finally, the recent works of the French School concerning tests of vitamin E action in man are very significant. H. Councille C. Harmay and M. Blondin (1953), H. Toulon, M. Blondin and H. Councille (1954) and H. Councille, C. Harmay and H. Rehh (1954) administered alpha-tocopherol in a single dose from 2 to 10 mg (!) to normal and diabetic subjects, with no consequences.

II. -- Description of the experiments

One group of 30 guinea pigs of an average weight of 250-300 g was divided into 5 groups of 6 guinea pigs each. The animals were kept in the same environmental and nutritive condition receiving the same food. We used confections of vitamin E of 300 mg per phial, administered intramuscularly, which was supplied for us by the firm of Bracco Merck, to which we extend our thanks.

Group I. -- The six guinea pigs were subjected to treatment with 100 mg vitamin E per day for 10 days. The guinea pigs showed no signs of disturbance during the treatment. At the end of the treatment there was noted an increase of weight of 25 to 50 g per guinea pig. Fragments of the muscle, kidney, liver, adrenal, heart and spleen were withdrawn, fixed in formalin and stained with hemetoxylin and eosin. Besides this, we also withdrew other pieces of the liver, heart, muscle, which were fixed in alcohol and stained according to the method of Best for purposes of the glycoge analysis; a fragment of the nervous substance of the encephalus was also withdrawn, fixed in alcohol and stained according to the Nissl method.

Group II. -- The six guinea pigs were treated with 300 mg vitamin E per day for 10 days. In these animals as well, we noted no signs of disturbance during the treatment. At the end of the administration we noted an increase in weight of from 30 to 50 g per guinea pig. Samples of muscle, kidney, adrenal, spleen, heart and liver were withdrawn, fixed in formalin and stained with hemetoxylin and eosin. Some fragments of liver, muscle, and heart were fixed in alcohol and stained according to the Best method for glycogen analysis. A piece of diseased encephalic substance was fixed in alcohol and stained according to the method of Nissl.

Group III. -- The six guinea pigs of this group were treated with 3 different doses: two guinea pigs were treated with 600 mg in a single dose which was not repeated; two others were inoculated with 900 mg vitamin E in a single dose (group IV). The other two guinea pigs were treated with 1,200 mg vitamin E in a single dose (group V). All six of the guinea pigs were sacrificed after four days following the administration. All six had survived; the guinea pigs treated with doses of 600 and 900 mg presented no signs of disturbance worthy of notice; the body weight remained nearly invariable; the guinea pigs treated with 1,200 mg presented a slight roughness of fur and a slight decrease in vivacity; their weight remained invariable. After

four days, samples of kidney, adrenal, spleen, heart and liver were withdrawn, fixed in formalin and stained with hematoxylin and eosin. Sections of liver, muscle and heart were also withdrawn and fixed in alcohol, and stained according to the Best method for glycogen analysis. Sections of encephalic matter were fixed in alcohol and stained with the Nissl method.

We are reporting here the results of this first group of experiments. The histological analyses were performed on all the animals treated; however, for purposes of brevity, we are reporting here, for every organ, one single bioptic result per group, since the histological findings are nearly identical and superimposable.

III. -- Description of the histological findings

Group I -- (100 mg intramuscularly per day for 10 days)

Muscle. -- There is a prevalence of compacted muscular bundles with fibers rich in sarcoplasma, and regular distributio of nuclei. In some spots there is a slight thinning of the single fibers, with slight edema of the interfibrillary spaces, a moderate proliferation of the nuclei of the interspaces; in the transverse sections one has the impression of a multiplicati of the nuclei and the single fibers.

Spleen. -- rather hypertrophic follicles, with well-conser centerfollicular arteries; notable blood engorgement of the sinu with vasal ectasias and rich reticular endothelial proliferation with marked necrophagial activity in the vicinity of the numerou spreadings of blood. The fibers of the trabecula and cupula are slender.

Liver. -- Hepatic cells perfectly conserved, also in the Kiernan's spaces and around the centrolobular veins. Everywhere there is observed a certain hyperplasia of the Kupfer cells and the interstitial endothelia.

Heart. -- Some miocardial fibers appear to be thinned, and reveal a certain hyperplasia of the interstitial nuclei. On the whole, however, one only rarely observes sections of turbidity of the fibers, and the general picture is nearly everywhere that of a well-conserved miocardium, even though there predominates a characteristic hyperplasia of young interstitial cells.

Adrenal. -- Thickness of the glomerular zone is diminished and it is poor in nuclei; the cells of the most exterior layer o the fasciculate appear here and there to be degenerated, while those of the other layers reveal a moderate lipoid content. The reticular zone is rather reduced. The medullar is of normal aspe

Kidneys. -- The majority of the convoluted tubules is norm only a small number showing marginal epithelia which are a bit squashed, with flaking cytoplasm, and the loss of some nuclei, glomeruli of form, and normal dimensions, alongwith some albumin in the Bowman spaces; afferent and efferent vessels normal.

Group II -- (300 mg per day intramuscularly for 10 days)

Muscle. -- Rare zones of palire of some groups of fibers i apparent degeneration, in contact with other well-conserved fibers.

Spleen. -- Rather small follicles, but with numerous germi

native centers; dilated sinuses with large empty spaces. Slight reticular endothelial reaction.

Liver. -- Some zones of the parenchyma, especially in the vicinity of the centrolobular zones, reveal cytoplasmic cells empty of content, or with a fragmented content. Some cells are deprived of there nucleus, some have an interrupted cell membrane and round nuclei, with thick chromatin, sometimes in a state of pyknosis. Here and there there are noted, especially in the vicinity of the vessels, all ectasic and replete, groupings of cells of the reticular endothelium, with nuclei with clear, vesicular rods. On the whole there are signs of a degenerative ailment of the parenchyma.

Heart. -- There are frequent zones of turbidity, of the type of wax degeneration or turbid swelling, where the normal fibrillary woof has disappeared, and the single fibers present signs of petrissage or fragmentation of substance. In some tracts, the interfibrillary spaces are somewhat enlarged. These lesions are more then frequent in the vicinity of the veinous branches of the coronary.

Adrenals. -- The glomerular zone is squashed, rather poor in cells; the most external layers of the fasciculata are rather faded; the rest of the parenchyma is normal.

Kidneys. -- Signs of slight ailment in some convoluted tubes, with swollen flaking epithelia. The entity of the glomerula is perfectly conserved, some of them showing rather ample Bowmann spaces and some ectasial capillary anses, with diapedesis of a few red corpuscles.

Group III -- (600 mg intramuscularly in a single dose)

Muscle. -- Normal structure.

Spleen. -- Sparse follicles; under light, the centrofollicular arteries are seen to be closed by swollen endothelia of the intima, which is thickened, and there are signs of a slight hyperemia the pulp reaction is moderate. The sinus and the fibrous woof are normal.

Liver. -- The majority of the cells appear to contain an acqueous cytoplasma, which is not stainable with eosin, and is in a moderate state of disease. The nuclei are rather well conserved, and only about 1/5 of them show signs of ailment. Well-conserved columns of hepatic cells are revealed in the margin of the lobules, in the vicinity of the portal spaces (see fig. 1).

Heart. -- There is a moderate edema of the interfibrillary spaces. The structure of the miocardium is well-preserved, and all the fibers appear well colored.

Adrenals. -- Half of the fascicular and glomerular zones contain faded cells with undefined margins and nuclei which are often pycnotic. The cells of the glomerular layer are functioning well.

Kidneys. -- The tubules appear normal; the glomerula are numerous.

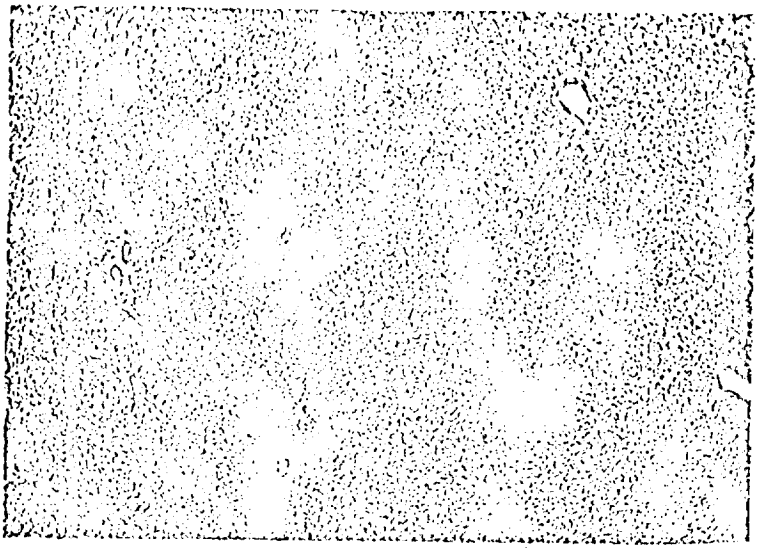

Figure 1. -- Guinea pig treated with a single dose of 600 mg vitamin E. Liver. Hematoxylin-eosin. x 48. Vast areas of fatty degeneration of the hepatic parenchyma. Columns of hepatic cells of colored cytoplasma are verified only in the vicinity of the portal spaces.

Figure 2. -- Guinea pig treated with a single dose of 900 mg vitamin E. Liver. Hematoxylin-eosin. x 200. Marked ailment of all the cells, many of which have lost the nucleus and reveal an interrupted membrane. Diffuse rhinosis.

Group IV -- (900 mg intramuscularly in a single dose)

Muscle. -- Normal structure.

Spleen. -- There is a richness of follicles; the pulp is full of reticulo-endothelial elements. Marked hyperemia. The fiber woof is normal.

Liver. -- About 2/3 of the parenchyma reveals degenerative factors similar to those described for the other animals, above all in the zones around the centrolobular veins (fig. 2).

Heart. -- In the subepicardial zones there are areas of the parenchyma which are discolored, with an irregular disposition of the fibers, which appear fragmented and with sparse, dark and amorphous nuclei.

Adrenals. -- The glomerula appears rather squashed, with slight hints of hyperplasia toward the interior. Many of the cells of the most external layer of the fasciculata are in a state of inflation. Nothing unusual in the medullary region. (fig. 3).

Kidneys. -- Nothing unusual in the tubules and glomerules.

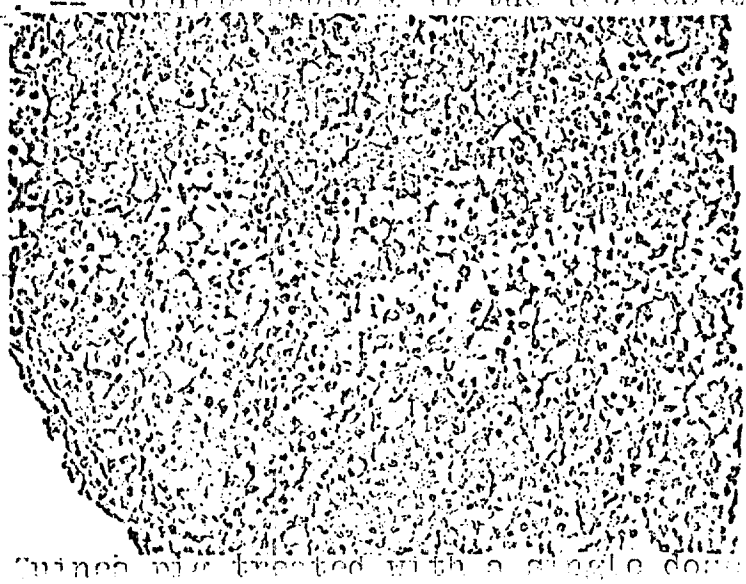

Figure 3. -- Guinea pig treated with a single dose of 900 vitamin E. Adrenal glands. Hematoxylin-eosin. x 200. Marked vacuolization and inflation of the cells of the fas[illegible] lay[illegible]. The glomerular layer is nearly unrecognizable.

Group V -- (1,20[illegible] intramuscularly in a single do[illegible]

Muscle. -- Normal structure.

Spleen. -- There is a [illegible] vascl ectasia with [illegible] [illegible] The pulp is rich [illegible] histioid elements [illegible] ith intense [illegible] activity.

Liver. -- The degenerative ailment of the lobules, where the reticular woof can be recognized, is quite marked. The cellular contours are preserved, but the cytoplasms appear faded, sometimes fragmented, and the nucleus is often eccentric-pyknotic. There is also seen a certain hyperplasia of the interfascicular endothelia and the Kupfer cells.

Heart. -- There are zones of paleness of rather moderate extent, while the weft of the syncytium is well-preserved.

Adrenals. -- The glomerular zone appears to be deprived of elements, and reveals signs of marked ailment of the cells of the

reticular layer, which appear reduced in size, rarefied, deformed, with a cytoplasm of homogenous eosinophile, but with a pyknotic nucleus. (Fig. 1).

Kidneys. -- Normal structure, nothing unusual in the tubules and glomerules.

In the sections stained according to the Best method, the results of analysis were as follows:

Group I -- Liver: no glycogen; Heart: no glycogen; Muscle: present (+).

Group II -- Liver: abundant glycogen (+++++); Heart: absent; Muscle: present (++).

Group III -- Liver: abundant glycogen (++++); Heart: absent; Muscle: present (+).

Group IV -- Liver: glycogen barely present (+); Heart: absent Muscle: present (+).

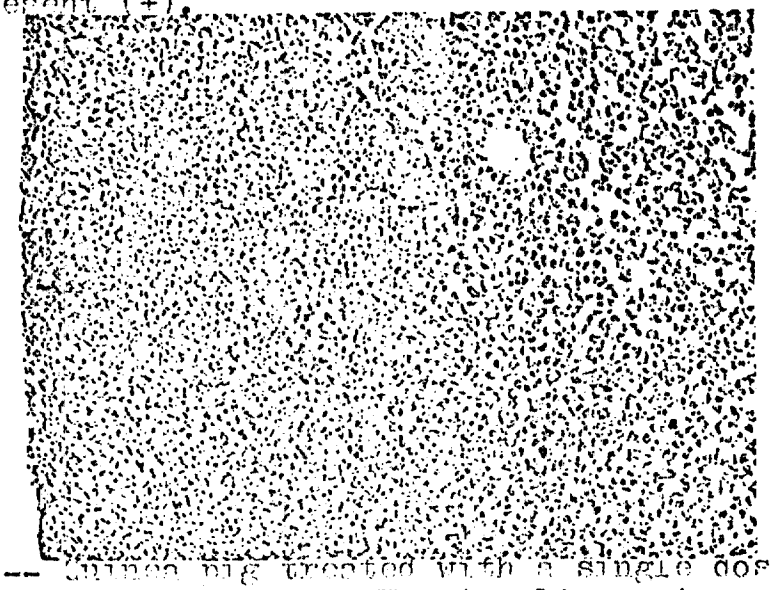

Figure 4. -- Guinea pig treated with a single dose of 1,200 mg vitamin E. Adrenal glands. Hematoxylin-eosin. x 100. Crispatio of the cells of the reticular layer, in the grip of pyknosis. Regressive (vacuolization) factors in the cells of the fascicular and glomerular layers as well.

Group V -- Liver: glycogen always abundant (+++); Heart: absent; Muscle: absent.

In the sections stained according to the Nissl method, the encephalic substance revealed the following histological alterations:

Group I -- Ganglionic cells well-preserved, with a well-evident nucleus. Rather rarefy neuroglia with loose mesh. Normal meninges.

Group II -- Moderate edema of the white matter. Ganglionic cells moderately preserved, though with dark, intensely basophilic cytoplasm and hyperchromatic nucleus. Hints of perivascular neurophagia.

Group III -- Intense edema of the white matter; ganglionic cells ample but corrected, with well-evident cytoplasm with

dense chromophile granules in irregular masses, and zones of perigenglion edema.

Group IV -- Manifest edema of the white matter; numerous ganglionic cells with evident chromphilic substance. Nuclei well-evident.

Group V -- Marked edema. The ganglionic cells are not very numerous; cytoplasm faded and clear; nucleus well-evident, some nuclei appearing reduced in volume and surrounded by edema. Some phases of tigrolysis with cellular destruction and phenomena of fagocytosis are noted. The overwhelming majority of the ganglionic cells still, however, has an evident nucleus, even if the cells reveal a slight degree of ailment.

In another group of experiments conducted on 2 groups of animals (6 male guinea pigs and six female guinea pigs), we wished to study the glands of internal secretion. We administered 600 mg Merck evion per day for 3 days, and then 300 mg evion per day for 2 days. After another 3 days, we sacrificed the guinea pigs. Another group of guinea pigs (three male and three female) was sacrificed for the histological comparisons.

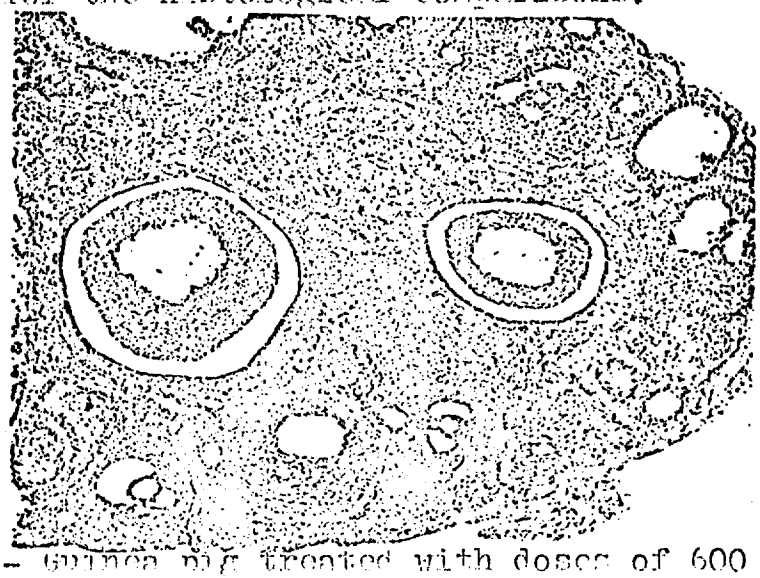

Figure 5. -- Guinea pig treated with doses of 600 mg vitamin E per day for 3 days, and then 300 mg per day for 2 days. Ovary. Hematoxylin-eosin. x 48. Intense follicular maturation in various phases, with facts of luteinization of the stroma.

These are the histological findings verified and synthetized here for the group of animals:

Hypophysis. -- Notable prevalence of acidophilic cells; scaling of some cell columns, while others are rich in cells with numerous hypersecretive images. On the whole, intense acidophile secretion with initial phases of functional exhaustion following intense stimulation. Nothing in the nervous lobe.

Thyroid. -- On the whole, the thyroid reveals rather distended utricles, delimitated by the cubic epithelium, which is sometimes retracted, full of colloid and acidophilic fluid; subtle glandular cords with a narrow lumen, poor or nearly deprived of colloid, and with a rather high epithelium. On the whole, presence of normal

Pancreas. -- Islets rather more numerous, some of which are larger than usual; the insular stroma is much enlarged and often thickened. Prevalence of beta cells, with hyperplasia of the stromal nuclei; scarcity of alpha cells.

Adrenals. -- Moderate vacuolization and loss of cytoplasmic acidophile in the cells of the glomerular zone. Signs of ailment in the cells of the reticular zone, which are reduced in volume and have a pyknotic nucleus. Ectasia and repletion of the capillaries. Dichromasia of the majority of the cells of the rest of the gland.

Testicles. -- Convoluted and rectal tubules rich in cells. Spermatogonia in single and double layers, rather large. Intense spermatocytic maturation and production. Leydig cells voluminous, in moderate numbers. The aspect of the testicle is that of a gland undergoing intense generative activity.

Ovaries. -- The ovarian parenchyma shows the presence of numerous follicles in various stages of evolution. There is a tendency toward an active maturative capacity of the some follicles. In fact, some of the oophorous follicles reveal cells of the granulosa deposited in numerous layers, so as to close off the follicular cavity, while in some the oophorous heap is quite visible; in the majority, this is not perceived. The granulosa, in the phase of advanced maturation as a result of ovulation, is seen to be thickened to such a state that it occupies the entire light of the follicular cavity itself.

The theceal strata, in particular the theca interna, follow, both as concerns the evolution and the blood spattering, the maturative progress of the follicle.

In the richly vascularized ovarian stroma, in which the fibrocitary evolution of the interstice is very evident, vast areas composed of vesiculous cells (with cytoplasm rich in vacuoles) with vanished borders and with a roundish nucleus, either in the center or shifted to the periphery, in which the chromatin is deposited in small granules, can be found. These aspects can in part be attributed to yellow bodies in the process of regression, but very often, due to their irregular arrangement and their diffusion, they can be easily interpreted in the sense of a stromal luteinization.

On the whole, the findings described above indicate an intense ovarian stimulation, which takes place because of a rapid and plural follicular maturation corresponding to an intense stromal luteinization (fig. 5).

IV-- Discussions of the analyses

From the examination of our experiments we derive above all the optimum tolerance which the guinea pigs demonstrated in regard to strong doses of the vitamin used. In fact, in the group treated with 100 mg per day for 10 days, the guinea pigs revealed a substantial increase in body weight (25 to 50 g), without any particular manifestations. The guinea pigs of the second group, which received 300 mg per day for 10 days, demonstrated identical behavior; the increase of body weight was a little more moderate (10-30 g). In groups III and IV, which received

showed no signs of even minimal disturbance in the four days for which they were kept alive; the weight remained stationary. Only the guinea pigs treated with an "impact" dose of 1,200 mg showed signs, immediately after the injection, of general pain, with roughness of the fur and little vivacity; however, the weight remained invariable.

Vitamin E initially was put to use only for its action, considered specific, on the gonads, but subsequently it was revealed that it participates in more than one physiological process, where once it was not even suspected that its action extended. These indications were revealed, for example, in various animals, by the conditions of deficiency in this vitamin, in as much as lesions were found in the most diverse organs and systems (E. Sbarigia, 1950). More accurate investigations (E. Sbarigia, 1951) have placed in evidence the possibility that vitamin E is one of the most important factors in the metabolism of the tissues, where it develops an essentially trophic function (P. Prosperi, 1950); however, its mechanism of action is far from being completely clarified. In fact, for example: even regarding that which is related to the nervous system, the best results seem to be those obtained in the treatment of the chorea (although many nervous lesions have been treated with this vitamin, but with no or slight effectiveness), but they still did not clarify the vitamin's metabolic action.

Vitamin E is involved in both the protein metabolism and the glycidic metabolism, as well as the lipidic. In regard to the protein metabolism, vitamin E seems to produce in the organism a better utilization of the amino acids. As concerns the glucidic metabolism, it seems to develop an action rather more far-reaching than that inherent to the sole production of muscular energy: in fact, it might also have an insulin-like action when connected with the activity of the phosphatases.

More marked is the action of vitamin E as concerns the glycogen, though the interpretation of the mechanism of action on the glycidic metabolism is not very clear (P. Prosperi and F. Ragazzini, 1950); in fact, it seems that after the administration of vitamin E, there is seen initially, in the liver, a diminution of glycogen, and subsequently a greater deposition, and besides this, there is a constant increase of glycogen in the stiated muscles and in the heart (P. Prosperi). More precise data on the behavior of the glycogen, revealed both histologically and chemically, in relation to the administration of vitamin E, are the following (P. Prosperi, 1950): in rats deprived of the vitamin there is seen a decrease of hepatic and cardiac glycogen (C. Bomskov and K. Von Kapulla, 1941), as well as muscular (the same authors; H. Goettsch and A. Pappenheimer, 1941). C. Bomskov also claims that a single injection of vitamin E after 36 hours leads to a decrease of glycogen. H. Butturini and L. Bagni (1942), treating guinea pigs with 3 mg per day for one month, saw an increase of the muscular, cardiac, and hepatic glycogen. M. Sposito and C. Nava (1942), chemically dosing the hepatic, cardiac and muscular glycogen in guinea pigs which had received 30 mg per day for 30 days, saw a decrease in the liver and the heart, and an increase in the muscles.

With a single dose of 60 mg, P. Prosperi (1950) found that the hepatic glycogen, after an initial decrease, increases in an unforeseen manner after 4 hours; that of the miocardium and the muscles, disappeared in the first few hours, reappears in the following ones; maintaining the treatment for 5 days, he found an increase of the glycogen in both the muscles and the liver and miocardium.

The pictures which are obtained in the course of vitamin E deficiency (G. Sbarigia, 1950), which coinvolve the muscles (in which there is verified an increase of the peroxidasic substance) with lesions of apparently pure miogenic origin, the heart (with more important and more constant alterations), the gonads (to which we will return in greater detail later), the adrenal cortex (perhaps because of a reflex of the lesions of the gonads, as happens in the case of the hypohysis), the adipose tissue (which reveals brownish pigmentation), the teeth (which reveal signs of depigmentation), demonstrate that this vitamin indubitably interferes in the intimate metabolism of the various tissues.

Experiments on the lesions of hypervitaminosis E have been very scarce (E. Sbarigia, 1950), and only concern investigations of the testicles and ovaries; findings concerning the other parenchymas are scarce. In normal conditions, when high doses of this vitamin are administered, it seems to be stored principally bey the liver; according to other authors, the major amount of vitamin E is contained in the adrenal capsules, which should be considered the regulatory center of the distributi of this vitamin.

Relative to weight, the testicles and the uterus are the organs most rich in vitamin E. At the recent 3rd International Congress on Vitamin E (Venice, 1955), H. Weinsen reported that the hypophysis is the organ with the highest content of vitamin E (90 mg %), followed by the adrenal (34 mg %).

L. Weissberger and R. Harris (1943), using high doses of vitamin E verified an increase of the phosphorus metabolism in the bone of rats.

Let us now take into consideration the alterations verified in the various parenchymas examined (liver; kidneys; spleen; muscle; heart; brain) in our experiments. In the first group (which received 100 mg per day for 10 days), the histological analyses revealed a complete absence of lesions in all the organs and parenchymas mentioned above.

In the second group (which had received 300 mg per day for the same period of time) there was noted a slight degree of ailment of the hepatic cell and the miocardial cell, with slight edema of the nervous substance. In the other groups there is a progressive development, with the increase of the dose of vitamin injected, to more outstanding lesions, always most pre-eminent in the liver, the miocardium and the brain.

In the third group (which received one "impact" dose of 600 mg), the hepatic cellular ailment is more outstanding (especially around the centrolobular veins); in the miocardium

there is evidence of interfibrillary edema; edema of the white cerebral matter is marked. In the fourth group (which received an impact injection of 900 mg), around 2/3 of the hepatic parenchyma demonstrates signs of degenerative ailment: the ailment of the miocardium, with slight cellular discoloration and phenomena of fragmentation of the fibers is equally evident. The nervous substance presents a picture of more outstanding edema. In the fifth group, finally (which had received an impact dose of 1,200 mg), the most serious lesions were found; in the liver one can only recognize the cellular controus and the fibrillary woof, while the nuclei are pyknotic and eccentric. Equally marked is the ailment of the miocardium and that of the nervous substance(the nervous cells reveal phenomena of tigrolysis). The spleen as well, undisturbed in the preceding groups, shows notable hyperemia and some spreading of blood.[1]

The behavior of the glycogen in the parenchymas in which it was analyzed (liver, heart, muscle) was various; in the first group it was verified in a moderate quantity only in the liver and the muscle; in the second group, on the other hand, though always being absent in the miocardium, it is increased in the liver and the muscle; in the other groups, treated with impact doses, the glycogen was always absent in the miocardium, scarce in the muscle, and present in a moderate quantity in the liver.

From the data first reported, we note a difference in behavior of the various parenchymas: the most interesting were the liver and the miocardium; the nervous tissue less so; the muscle least.

Very probably, this difference of behavior in normal conditions can be imputed to their diverse metabolisms in regard to vitamin E (and consequently also to glycogen; in fact, the investigations of the glycogen also demonstrated a different behavior for the various parenchymas); it seems logical to me that the response of the various parenchymas is therefore also different in relation to high or impact doses of vitamin E.

We have seen that vitamin E seems to be stored principally in the liver; if this is true, this phenomenon can explain the progressive ailment of the parenchyma with the increase of vitamin E doses. This ailment is probably due to either an excess of the dose, or to a defect in digestion; we cannot say more, not knowing how this vitamin is metabolized. The interpretation of the miocardiac ailment is difficult; can it be presumed that it is related to the verified lack of glycogen?

The lesions of the nervous system can be due to the play released by the vitamin in the metabolism of the tissues (consider its effectiveness in the cure of the chorea). As regards the glycogen more precisely, the following can be said: after the observations of the varied influence of different doses of vitamin E on the accumulation or disappearance of glycogen in

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[1] We wished to verify whether the regressive alterations of the parenchymas were not to be imputed to the administration of too high quantities of oil contained in the phials of Evion. Administering to four groups of two guinea pigs each sterile olive oil intramuscularly in the respective quantities of 2, 4, 6, and 8 cc. we verified, upon examination of the sections, moderate foci of

the various parenchymas examined, we maintain that the results which we obtained are probably due to the diverse metabolism of glycogen in the various parenchymas. The observations already made, that while glycogen disappears with the use of low doses, it reappears with the use of larger doses, are in any case reconfirmed. The behavior upon use of impact doses is not clear (there is a lack of comparative elements in the literature on this topic).

It was logical to research the interference of vitamin E in the endocrine system.

In normal conditions, of note are the investigations of the gonads, which first gave encouragement for the use of vitamin E. As concerns other glands as well, H. Sowade (1940) found a slight thickening of the follicular epithelium in the thyroid; Greoss (cited by Prosperi) saw slight thyroidal hypertrophy; richness in cells, especially neutrophile, in the anterior lobe of the hypophysis; facts of follicular atrophy, reduction of the follicles, and sometimes increase of the yellow bodies in the ovaries; no lesions of the pancreas and adrenals.

In pathological conditions there has been verified: 1) in cases of vitamin E deficiency (Sbarigia, 1950): in the rat there are evident lesions in the germinal part of the testicle, while the endocrine part remains intact; in the ovary, rather scarce lesions, or barely apparent ones (greater quantity of connective, less marked pigmentation, decrease of the neutral fats and the appearance instead of linides). No hypophysial lesions were found (these appear instead when the testicle or the ovary is injured.). Pio (1929) saw, in rats with vitamin E deficiency, lesions of the genital organs, the thyroid and the adrenal. 2) in conditions of hypervitaminosis (Sbarigia, 1950): in female rats, with strong doses, Reiss (1941) found a decrease of the number of yellow bodies, with a decrease in the weight of the organ; in the small field mouse, upon use of high doses, he found no lesions of the testicle, while in the ovaries there was suppression of the formation of primordial follicles and death of the eggs in the follicles, in the process of maturation.

The results of our experiments were the following:

Hypophysis: intense acidophile secretion with signs of initial functional exhaustion.

Thyroid: normal aspect, which presents on the whole alternate phases of activity.

Pancreas: greater number of islets, with prevalence of beta cells.

Adrenals: signs of ailment in the glomerular and reticular zones. (These alterations are more outstanding with the use of larger doses.)

Testicle: the gland is in the terminal phase of an intense germinative activity.

Ovary: reveals the tendency toward an active maturative capacity of the numerous follicles in various stages of evolution.

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of fatty degeneration only in the liver, interesting only in the layers immediately surrounding the branches of the superhepatic veins.

However, the behavior of the various parenchymas examined was differing. In fact, it seems that the thyroid least resisted the treatment. The hypophysis, on the other hand, reveals a state of active secretion which leads the gland to exhaust itself functionally; this exhaustion is obtained by direct action on the gland, or in relation to the lively activity of the testicle and the ovaries, or due to the state of ailment of the adrenal. For the pancreas, it is found that vitamin E led to the production of a greater number of islets, with an increase of the beta cells, that is, of those cells more involved in the glucidic metabolism. The adrenal reveals much evidence of ailment, which becomes more marked upon use of the impact dose of 1,200 mg. As has been seen previously, vitamin E is contained in great quantity in the adrenal; in this case, its ailment is due to an action of accumulation, or a defect in elimination , or as a phase of exhaustion in the face of stress. More outstanding and more specific are the alterations in the testicle when it is stimulated by all the doses used of vitamin E. The ovaries as well, with the high doses used, responded with an active maturative capability of the follicles, to which an intense luteinization responded. It therefore seems indubitable, as has already been demonstrated by other authors, that vitamin E has a specific action in the ovaries. However, in our experiments, the alterations already indicated with the use of strong doses of vitamin E were not verified.

Conclusions

In conclusion, it can be said that the high doses of vitamin E used by us in experiments on the guinea pig let to signs of ailment of some parenchymas (especially the liver, miocardium and brain) and some endocrine glands (adrenal), while other glands of internal secretion were stimulated either in a moderate manner (pancreas), or in an outstanding manner (hypophysis, testicle and ovary).

If we take into consideration the fact that the doses used in these experiments, already very high for the guinea pig, compared with similar doses per kg of weight in man , would reveal entirely unreal values in therapy with vitamin E, we can conclude that the therapeutic use of vitamin E grants margins of security such that they allow the ample multiplication of the doses actually used.

But the delicate problem of clinical hyperdosage of vitamin E must be confronted considering also the biological action of alpha-tocopherol. In the recent 3rd International Congress in Venice, the outstandingly oxido-reductive action of alpha-tocopherol was unanimously recognized (with H. Dam being its most ardent supporter). E. Pierangeli, who was the coordinator of one of the most interesting days of the Congress, concluded the discussion by explaining the concept that such an action of vitamin E must be understood not as being exercised on single organs or systems, but on the level of all the cells universally, and he added that the ante-hypophysial cells, as the most differentiated, must resist more than the others this anti-oxidatin action.

This induces us to reflect that, taking into account the high vitamin E content of the hypophysis and the adrenals (which the second most involved glands in our research), it is necessary to be careful in the use of hyperdoses which, if harmless under the general conditions of the organism, and useful in resolving particular dismetabolic situations, can nonetheless be harmful to some particular tissues.

Summary

The authors treated five groups of guinea pigs with high single and fractionated doses of vitamin E, administered intramuscularly.

Following the use of large doses, even if administered in a single dose, degenerative lesions in the liver and, to a lesser degree, the miocardium and the encephalic substance, were noted.

A more detailed study in regard to the behavior of the endocrine system in conditions of hypervitaminosis E revealed serious phenomena of damage in the adrenal glands, and increterory stimulation in the pancreas, and above all in the hypophysis, the testicles and the ovaries.

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VITAMIN E IN FOODS AND TISSUES*

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Quantitative determinations of the amount and kind of tocopherols ingested and stored by different species of animals have revealed an interesting similarity and uniformity in the physiological utilization of vitamin E. Apparently, α-tocopherol is preferentially absorbed and deposited in tissues of chickens, cows, and humans. This strongly suggests that daily intakes and nutritional requirements of vitamin E should be expressed in terms of d, α-, rather than total, tocopherol.

In one experiment, laying hens were fed pure tocopherols at increasing levels as a supplement to a vitamin E-free diet. One object was to study the relation of level of tocopherol in the diet to its deposition in eggs. Another was to see whether evidence of *in vivo* conversion of non-α- to α-tocopherol would be found.

Pure, natural tocopherols, α-, γ-, or δ-, were given to hens by capsule in individual doses at levels of 100 up to 4,000 mg./week. The eggs were collected and tocopherol assays made on them for total and for γ- plus δ-tocopherols, α-tocopherol being estimated by difference.[1] When weekly supplements of tocopherol are plotted *vs.* tocopherol concentration in eggs (after 2 weeks on supplement), the latter appears to increase linearly with log dose fed. The hens on α-tocopherol supplement showed prompt and striking increase of tocopherol deposited in the eggs, whereas those given γ- or δ-tocopherol laid down proportionally much less tocopherol in eggs. For example, corresponding to the 400 mg. weekly tocopherol supplements, the respective concentrations in eggs were for α-, γ-, and δ-tocopherols—24.2, 5.7, and 2.3 mg./100 gms. fresh weight.

The relative efficiency of transfer of α-tocopherol to eggs by hens, as compared to γ- and δ-tocopherols, constitutes evidence of selective deposition in tissues of α- as compared to non-α-tocopherols by the animal body. Maximal values were calculated on a weekly basis. They were: α- 22.1 per cent, γ- 3.6 per cent, and δ- 2.0 per cent.

No evidence was found to show that there is *in vivo* conversion of non-α- to α-tocopherol. The δ-tocopherol-fed hen laid eggs with steadily increasing δ-tocopherol content as the level of supplementation was increased, while the γ-tocopherol-fed hen laid eggs which contained more than 90 per cent γ-tocopherol of total tocopherols at the higher levels of supplementation.

Increased deposition of tocopherol in cow's milk with increased supplementation was shown by a crossover type of experiment, wherein six cows each received six different levels of supplement. One supplement was a 60 per cent α-tocopherol preparation, and the other contained 90 per cent of γ- plus δ-tocopherols. Cows which received the larger supplements of α-tocopherol secreted much more tocopherol in the milk, while the non-α-supplements caused only slight increases. Milk from the former increased

* Communication No. 153.

in tocopherol content from 0.025 to 0.068 mg./gm. fat as the supplement was raised to 10 gm. total tocopherols daily. In the latter, the values were 0.022 to only 0.034 mg./gm. fat. This provides further confirmation of the selective deposition of α-, as compared to non-α-tocopherols by the animal body in its tissues and fluids.

Tocopherol values for all tissues of an adult male rat have been determined (TABLE 1). The rat received 1 mg. per day of α-tocopherol as a

TABLE 1

DISTRIBUTION OF TOCOPHEROL IN TISSUES OF AN ADULT MALE RAT*

Tissue	*Weight (gm.)*	*Tocopherol (mg./100 gm)*	*Concentration (mg./gm. fat)*	*Total tocopherol in the tissue (mg.)*
Blood cells	—	—	1.1	0.047
Suprarenals	0.04	34.0	0.7	0.014
Lungs	1.38	3.24	0.74	0.045
Spleen	0.58	5.1	1.1	0.030
Liver	10.6	2.52	0.51	0.269
Blood plasma	—	0.70	—	0.070
Gut	4.64	3.69	0.42	0.171
Kidneys	2.04	1.18	0.24	0.024
Thymus	0.49	1.7	0.7	0.008
Diaphragm	0.66	2.5	0.7	0.016
Heart	0.90	3.42	0.96	0.031
Penis	0.24	4.5	1.0	0.011
Seminal vesicles	0.72	2.6	0.7	0.019
Residue (skeleton Head, etc.)	91.0	3.55	0.57	3.23
Mesentery fat	5.85	6.0	0.071	0.349
Pelt	58.5	3.32	0.39	1.94
Muscle	86.0	1.33	0.54	1.15
Testes	3.18	2.26	1.05	0.072
Pancreas	1.62	5.48	0.20	0.089
Pituitary	0.01	90.	1.2	0.009
Central nervous system	2.49	1.62	0.16	0.040
Total				7.634

*Maintained on a vitamin E-free diet plus a supplement of 1 mg./day d,α-tocopherol.

supplement to an E-deficient diet. A total of 7.634 mg. was found in the rat. Pituitary and adrenal glands were very rich in tocopherol, having 90 and 34 mg./100 g., respectively. These high values for the single rat organ assays are confirmed by results found on pooled organs of other rats. Pituitary glands had 4 to 78 mg. tocopherols/100 g. and adrenals had from 26 to 332 mg. tocopherols/100 gm., fresh weight.

Of the remaining rat tissues, values on a fresh weight basis ranged from 5.85 mg./100 gm. for mesenteric fat to 0.70 mg./100 ml. for blood plasma. This concentration range is fairly close to that reported by Mason for tissues of rats maintained on low-E rations.[2] His range is 3.3 to 0.8 mg./100 gm. The total tocopherol in Mason's rat was estimated to be about 3 mg. in contrast to 7.6 mg. found here.

The tocopherol concentrations in rat tissues, per gram of fat, resemble

the values in human tissues, ranging from 0.1 to 1.1 mg. tocopherol/gram of fat.

Tissues from normal humans have been assayed for their total and γ- plus δ-tocopherol contents.[3] Alpha-tocopherol was estimated by difference. For a man, a total of 3.4 gm. of tocopherols, of which 91 per cent was α-tocopherol, was calculated. The corresponding values for a woman were 8.1 gm. total tocopherols—88 per cent α-tocopherol. Human tissues showed a fifty-fold range of tocopherol concentration when expressed on a fresh-weight basis. The tocopherol concentration range, expressed on the basis of extracted lipid, was less than eight-fold: 0.2 to 1.2 mg./gm. in both man and woman. That tissues of the two human subjects seemed to have 90 per cent α- of total tocopherols indicates that α-tocopherol is preferentially stored in body tissues, since the usual daily intake of tocopherol consists of about $\frac{1}{2}$ α- and $\frac{1}{2}$ non-α- forms, according to dietary calculation.

Alpha-tocopherol is probably also absorbed preferentially to non-α-tocopherols in humans. Two studies from our laboratory indicate this. Absorption curves were obtained for 5 normal male adults who took 500 mg. doses of pure natural α- and of pure natural γ-tocopherol at random intervals. Values were determined on 0.06 ml. serum, obtained by finger-tip puncture.[4] The absorption of α-tocopherol was increased over that of γ- in the 27-hour period measured (FIGURE 1). Blood from 18 normal subjects who had not

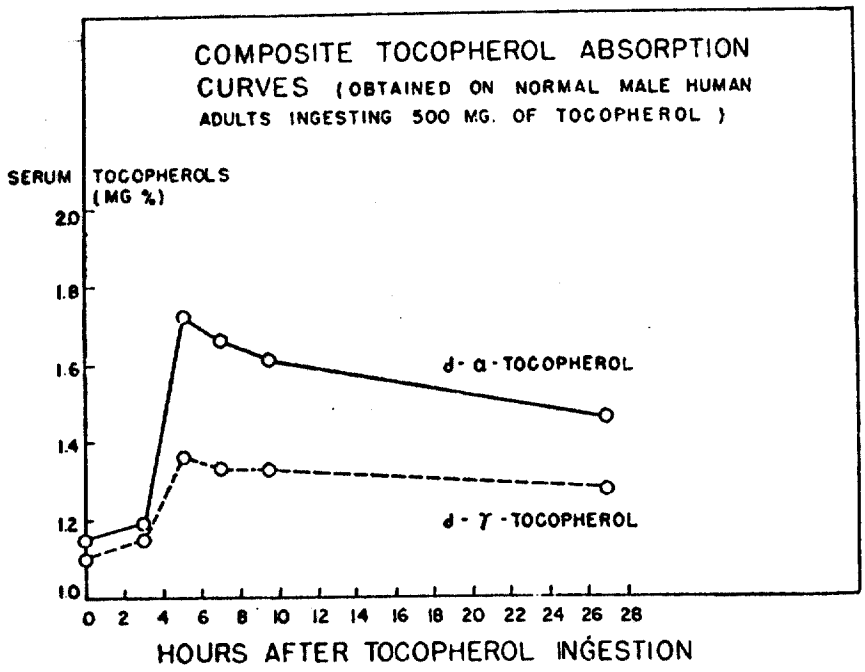

FIGURE 1. Comparative absorption of α- and γ-tocopherols by normal human adults.

received tocopherol supplements was assayed for total and for γ- plus δ-tocopherols. Total tocopherols averaged 1.00 mg. per cent for the series

and non-α-tocopherols only one-quarter of this. If α- and non-α-tocopherols occur in the diet in roughly equal amounts, this suggests that the former is more readily absorbed.

Estimates of dietary intake of vitamin E by humans throw some light on the probable human daily requirement. This should be expressed in terms of d,α-tocopherol or its biological equivalent, since the body seems to absorb and store it in the tissues with very much greater efficiency than it does the non-α-tocopherol forms.

With data on the total and non-α-tocopherol content of a variety of foodstuffs at hand, it has been possible to estimate the probable content of various diets in terms of α-tocopherol. Typical menus (3,000 Calories) which are adequate, as judged by current nutritional standards, have 10 to 25 mg. of α-tocopherol. The average daily per capita food consumption for the U. S. in a recent year, which yields a 3300 Calorie intake, provides 19 mg. of α-tocopherol.

Diets of a small series of women, who are subjects in a study of nutrition in pregnancy being conducted by Dr. N. S. Scrimshaw in Rochester, New York, have been evaluated for vitamin E. The α-tocopherol content ranges from 5.7 to 12.8 mg. per day.

In contrast, the α-tocopherol in numerous special diets, which are used for therapeutic purposes, amounts to less than 10 mg. (TABLE 2). Persons

TABLE 2
TOCOPHEROL CONTENT OF VARIOUS DIETS

Type diet	*Total tocopherols (mg.)*	*Alpha tocopherol (mg.)*
Low sodium,* 1,910 Cal.	9.32	7.22
Diabetic diet,† 1,600 Cal.	11.00	8.60
Reducing diet,‡ 1,000 Cal.	5.9 to 18.6	4.3 to 6.6
Fattening diet,‡ 3,000 Cal.	11.8 to 80.2	9.9 to 26.6

* Modern Medicine: 44. Feb. 5, 1949.
† WOLF, G. M. Dietotherapy, Clinical Applications of Modern Nutrition: 619. W. B. Saunders. Philadelphia and London. 1945.
‡ J. Amer. Med. Assoc. 139: 86. 1949.

subsisting on such diets for long periods of time might be expected to be in a poorer state of nutrition with respect to vitamin E than those who are very well fed.

The National Research Council has recommended a group of foods to be consumed daily which will supply adequate amounts of all recognized dietary essentials. The group of foods contains 5.7 mg. of α-tocopherol (TABLE 3), which is quite low as compared to the probable human daily requirement. It is doubtful that foods which would be eaten, in addition to these in the list, to round out caloric requirements would supply appreciably extra α-tocopherol unless a large amount of vegetable fat were included.

Loss of vitamin E in foods due to cooking would decrease the values just given. The amount of loss is variable. Deep-fat frying is very destructive. For example, losses of tocopherol in doughnut fat range from 70 to 90 per

TABLE 3
VITAMIN E CONTENT OF DIET RECOMMENDED BY NATIONAL RESEARCH COUNCIL*

List 1	Calories	Tocopherol content	
		total (mg.)	alpha (mg.)
Milk 1 pint	330	0.65	0.65
Egg, 1	80	1.00	0.60
Meat, Fish, or Fowl	225	1.10	0.95
Potato, 1 or more	200	0.14	0.14
Vegetables, 2 servings	75	1.00	0.50
Fruit 2 servings	150	0.50	0.50
Cereals and bread	500	3.20	2.40
Totals	1560	7.59	5.74

*National Research Council. Recommended dietary allowances: 16. Reprint and Circular Series. No. 122 1945.

cent. Potato chips contain 0.1 mg. tocopherol/gram of extracted fat, compared to the usual concentration of tocopherol in vegetable oils of about 1 mg./gm. Baking apparently destroys tocopherol to a lesser extent; extracted fat from a series of pies, cakes, and cookies had 0.25 to 0.76 mg. tocopherols/gram.

These considerations suggest that, under good dietary regimes, the human daily intake of d,α-tocopherol, derived from the diet, does not exceed 25 mg.

The experiments described here further indicate that there is selective deposition and perhaps absorption of α-tocopherol as compared to non-α-tocopherols for three species—cow, chicken, and man.

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Discussion of the Paper

DOCTOR K. HICKMAN: Referring to Dr. Quaife's data showing the analysis of a female human, I suggest that the results constitute an interesting discovery. That a man contains up to 5 grams and a healthy woman the enormous quantity of 5–10 grams of vitamin E (comparable with the quantity of cholesterol, 50 g.) is an exciting piece of information. One notes, too, that nine-tenths of the vitamin is in the adipose tissue and only one-tenth in what we may call the operating tissues. Dr. Quaife's analyses of food and Dr. Engel's measurements of efficiency of absorption show that the real daily intake is not more than 10 mg. The following conclusions, if not unescapable, seem to me to merit most careful examination.

(*1*) For a woman, the dosage ratio is about 1000:1; the body contains a three years' supply of vitamin E.

(*2*) The period of "half-adjustment" for the whole body is about two years.

(*3*) The period of half-adjustment of the operative tissues could be as low as two months.

(*4*) A short period of adjustment for the operative tissues would entail an even more extended period for the adipose deposits. This would parallel the clinical findings that certain E deficiencies respond rapidly while others resist years of treatment.

(*5*) It may be assumed that there is free interchange between the adipose tocopherol and the active-tissue tocopherol. One would expect to find a definite partition coefficient established between them.

(*6*) If the last point is true, a person putting on weight on a low E diet would dilute the tocopherol in the fat depots and this would actually extract vitamin E from the operating tissues. A woman putting on too much weight in pregnancy could rob the placenta and the fetus of α-tocopherol at the time that it is most urgently required. This might serve to explain the physicians' anxiety that the pregnant female shall curb her appetite.

(*7*) A person (or animal) taking exercise after a period of rest will consume fat, thus making vitamin E available to the musculature just when needed. Perhaps Dr. Quaife's discovery gives us a glimpse of Nature's control mechanism for presenting α-tocopherol to the active tissues?

DOCTOR ADAMS: Infertile women in my medical practice become fertile when given tocopherol. Obese women known to be sterile often regain fertility following the loss of weight by systematic dietary restriction. Therefore, in essence, the experiment suggested by Dr. Hickman on the repartitioning of vitamin E has been carried out.

DOCTOR C. MACKENZIE (*Department of Biochemistry, Cornell University Medical College, New York, N. Y.*): Within what cellular component does Dr. Quaife consider vitamin E to reside?

DOCTOR M. QUAIFE: Our results on the distribution of tocopherol in tissues give no indication concerning the location of tocopherol in the tissues. The expression of vitamin E per unit weight of lipid, as well as per unit weight of fresh tissue, was made arbitrarily merely to afford easy means of comparison.

THE EFFECT OF VITAMIN E ON MESENCHYME METABOLISM*

Experimental Investigations on Animals, Concerning the Effect of Alpha-Tocopherol Acetate (Vitamin E) on the Metabolism of Sulfated Mucopolysaccaride of Connective Tissue.

Rave, O. & al. (Medical Clinic and Polyclinic, Wilhelms University of Westfalia, Munster) . Z. Rheumaforschg. 30, 266-275 (1971).

1. Introduction

In numerous case histories involving changes in connective tissues, favorable effects of Vit. E therapies were observed. Successful treatments were reported in diseases belonging to the so-called "collagenoses", such as the Dupuytren contracture, induratio penis plastica /5, 8, 14, 17, 30, 32, 33/ and sclerodermis /26, 31/.

In certain skin diseases, such as e.g. keloid, ulcus cruris, psoriasis and chronic cystic mastitis, favorable results were obtained with Vit. E therapy, also in some vascular diseases, both of the organic and the functional type (M. Raynaud, Claudicatio intermittens 1, 32) and arteriosclerosis /41/.

Further, positive therapeutic results of tocopherol therapy are known with respect to muscular distrophy /5, 40/, fertility disorders /29/, icterus juvenilis intermittens (Meulengracht), in geriatry /22, 36, 37/ and in diseases of the mobility and supporting apparatus /5, 10/.

In spite of this wide field of application, ambiguity largely rules concerning the exact action mechanism of tocopherol /13/. At the present time, the relationships of Vit. E to the other vitamins /3, 15, 35/ and hormones /40/ 43/ and its importance as an antioxidant, are known /9, 38, 40/.

In the present work, therefore -- especially because of the favorable effects of Vit. E on diseases localized in connective tissue -- the effect of tocopherol on the metabolism of normal tissue and itssue subject to inflammatory alteration, is investigated.

2. Methods and Experimental Apparatus

2.1. General

All of the experiments were performed on male Wistar rats with an average body weight of approx. 150 g. During the experiments, all animals were kept in identical environmental and feeding conditions. The individual experimental groups consisted of 8 animals each. In the Vit. E treatment the preparation "Evion-Aquat" of the E. Merch Co., Darmstadt, a water-soluble alpha-tocopherol was used in intramuscular injections.

* Supported by the Nordrhein-Westfalen State Office of Research, the Bochum Miners Union and the Westfalen State Insurance Institution.

2. 2. Methods

In the in-vivo investigations the animals received one day prior to their death 1 mC ^{35}S sulfate ($Na_2{}^{35}SO_4$, free of carrier, sterile in a 0. 5 ml physiological NaCl solution), injected i. p. . Following the killing of the animals, the tissues to be examined were dissected out and processed in a previously described manner.

In the in-vitro investigations the tissue to be examined was comminuted and placed in an Erlemeyer flask with 5 ml of a ^{35}S sulfate-containing incubation solution.

To investigate the effect of Vit. E on the metabolism of connective tissue, a certain amount of Vit. E was added to the incubating medium in part of the flasks. The tissue sections in the rest of the flasks served to determine the specific activity of the sulfated mucopolysaccharides in cpm/ 100 millograms SMPS. In the illustrations, the results are presented in % of the controls (control values = 100%). A detailed description of the method and an accurate analysis of the incubating solution may be found in Junge-Hulsing /24, 23/.

2. 3. Experimental Order

2. 3. 1. Investigations of the Effect of a Single High-Dose Vitamin E Application on Mesenchyme Metabolism.

The experimental animals received a single subcutaneous injection of 100 mg of Vit. E. Another group of animals was left untreated for control purposes. The metabolic activity of the connective tissue was determined, based on the incorporation of ^{35}S sulfate in the SMPS in the heart, aorta, skin and cartilage tissue, on the 1st, 2nd, 3rd 5th, 10th and 18th day after the last application.

2. 3. 2. Investigation of the Effect of Different Vitamin E Doses on the Metabolic Activity of Connective Tissue.

To investigate the dosis dependence of the inhibition by Vit. E of mesenchyme metabolism, one group of animals received 10 mg, a second group 25 mg Vit. E per day by intramuscular injection over a period of 14 days. The control group remained untreated. The incorporation of ^{35}S sulfate in the SMPS of the connective tissue of the heart, aorta and cartilage was examined on the 1st day following the last application.

2. 3. 3. Investigation of the Effect of Vitamin E on Mesenchyme Metabolism in Vitro.

In the in-vitro experimental order, a group was given 2 mg or 5 mg Vit. E in the incubating solution. The ^{35}S sulfate incorporation in the connective tissue of the heart, aorta and cartilage was determined.

2.3.4. Investigation of the Effect of Vitamin E on the Growth of Granulation Tissue.

To investigate the effect of Vit. E on the growth of granulation tissue, 3 experimental groups were formed: one group was treated with 10 mg, another with 25 mg Vit. E, i.m., over a period of 14 days; the third group remained untreated as the control group.

On the 9th day after the start of the Vit. E application, all of the animals of the three groups received an implantation subcutaneously of two mg cotton pellets during ether narcosis (method of Meyer & al.). On the 6th day after the implantation (315th day after the start of the Vit. E pretreatment, 1 day after the last Vit. E application), the fresh weight and the incorporation of ^{35}S sulfate in the granulation tissue of the cotton pellet granuloma were examined.

2.3.5. Investigation of the Effect of a Vitamin E Pretreatment on the Non-specific Mesenchyme Reaction in the Heart and Aorta Triggered by Toxin Irritation.

In order to study the effect of Vit. E on the local mesenchyme reaction following the administration of a toxin, three experimental groups were again formed: one control group remained untreated, one group received staphylococcus toxin (1/25 i.u.) injected i.p., the third group received toxin in the same manner and dosage and, additionally, 25 mg Vit. E over a period of 14 days.

One group received 25 mg Vit. E i.m. over 14 days and 1/25 i.u. of a staphyloccous i.p. on the last day of the Vit. E pretreatment, the second staphyloccous toxin only in the same manner and dosage, the third group remained untreated as the control. The incorporation of ^{35}S sulfate in the SMPS of the connective tissue of the heart and the aorta was determined 24 and 48 hours after the injection of the toxin.

3. Results

3.1. Effect of a Single High-Dosage Vitamin E Application on Mesenchyme Metabolism.

As shown by the investigation (Fig. 1), a single subcutaneous injection of 100 mg Vit. E in the connective tissue of the heart, skin and aorta results in a longterm inhibition of the rate of incorporation of ^{35}S sulfate, which could be detected even on the 18th day after the application of Vit. E. This inhibition of the metabolic activity of the SMPS was strongest in the connective tissue of the heart, where it declined between the 3rd and 10th day to values less than 50% of normal. In the connective tissue of the aorta, skin and cartilage fundamentally similar but less pronounced alterations of the SMPS metabolism were observed.

3.2. Dependence of the Inhibition of Connective Tissue Metabolism on the Magnitude of the Vitamin E Dose Administered.

Daily intramuscular injection of 10 mg Vit. E over a period of 14 days resulted in a clear inhibition of metabolic activity 24 hours after the last

application on 60% of the controls. The application of 25 mg Vit. E per day over the same period yielded only a slightly more intensive inhibition of ^{35}S sulfate incorporation rates in the SMPS of the connective tissue of the heart, aorta and cartilage (Fig. 2). This inhibition of mesenchyme metabolic activity was again most apparent in the connective tissue of the heart.

FIGURE 1. Change in Mesenchyme Metabolism of the Heart, Skin, Aorta and Cartilage After a Single Subcutaneous Injection of 100 mg Vitamin E: Inhibition of ^{35}S Sulfate Incorporation in the SMPS of Connective Tissue to the 18th Day Following Application. Control animals were not treated.

Key 1. Specific activity ^{35}S-cp, /100 SMPS in % of controls; 2. Heart; 3. Aorta; 4. Skin; 5. Cartilage; 6. Controls; 7. Days.

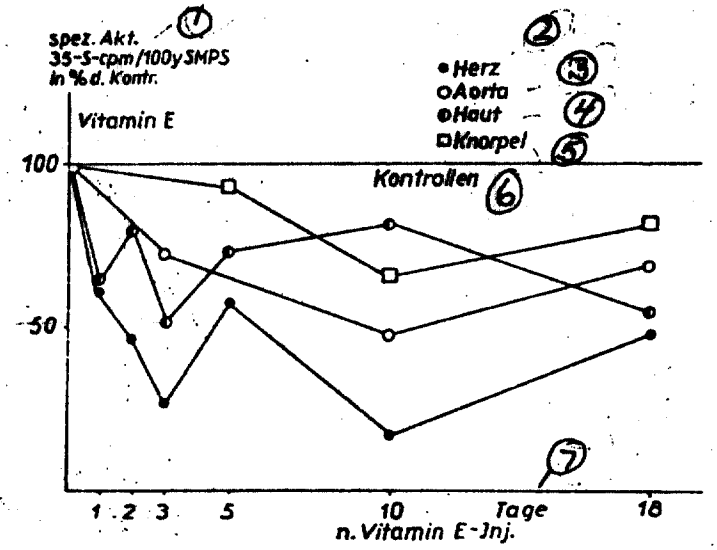
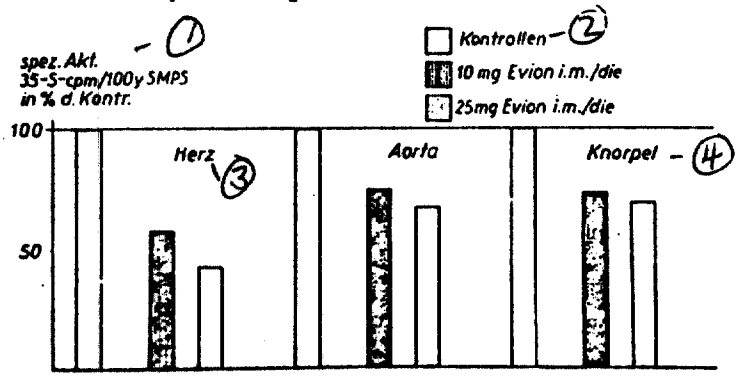


FIGURE 2. ^{35}S Sulfate Incorporation Rate in the Heart, Aorta and Cartilage After the Intramuscular Injection of 25 mg or 50 mg Vitamin E Over 14 Days. Control Animals Were Not Treated. One Day After the Last Administration, All Three Organs Showed a Clear Inhibition of Specific ^{35}S Sulfate Activity on the SMPS of the Connective Tissue.

Key 1. Specific activity ^{35}S-cpm/100 SMPS; 2. Controls; 3. Heart; 4. Cartilage.

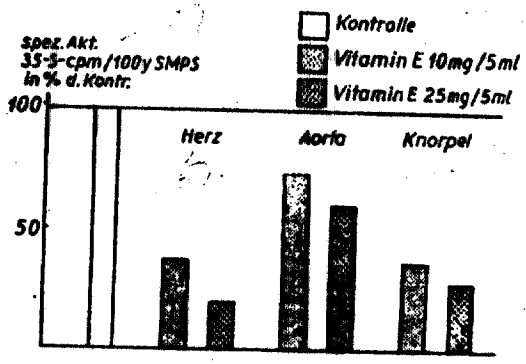


3.3. Effect of Vitmain E on the Connective Tissue Metabolism of the Heart, Aorta and Cartilage in In-Vitro Experiments.

Effects of Vit. E were detected under in vitro experimental conditions; they were dependent both on dosage and on the organ. If the ^{35}S sulfate incorporation rate in connective tissue of the heart of controls is set equal to 100%, the rate of incorporation of ^{35}S sulfate under the effect of 10 mg Vit. E/ml of the incubating solution amounted to 40% of the controls; with the addition of 25 mg Vit. E/ml of the incubating medium, ^{35}S sulfate incorporation rates were reduced to 20% of the controls. Corresponding values in the investigation of the aorta and cartilage amounted to 75 and 60% and 35 and 28% of the untreated controls (Fig. 3).

FIGURE 3. Determination of the Specific ^{35}S Sulfate Activity of the SMPS of the Connective Tissue of the Heart, Aorta and Cartilage in Vitro Following the Addition of 2 mg or 5 mg Vitamin E per ml of Incubating Medium. Control Values Were Determined After Incubation In a Solution Without Vitamin E.

Key: 1. Specific Activity ^{35}S cpm/100 y SMPS in % of controls; 2. Controls; 3. Heart; 4. Cartilage.

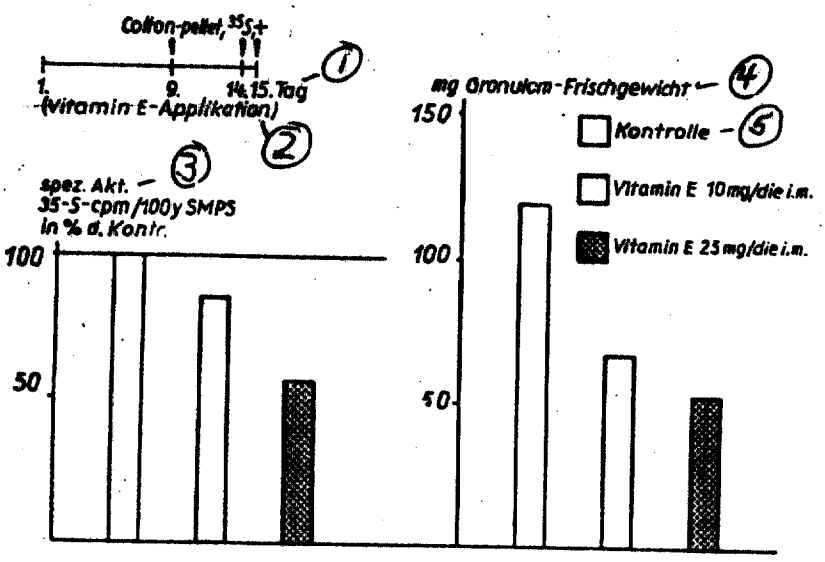


3.4 Effect of a Vitamin E Application on the Growth of Granulation Tissue.

The investigation of the growth of cotton pellet granuloma under the effect of Vit. E treatment showed that measured by the fresh weight of granuloma and by the incorporation of ^{35}S sulfate in the SMPS of connecting tissue -- the growth of granulation tissue is clearly inhibited. This effect was observed even with a dose of 10 mg Vit. E per day. An increase in the daily dose to 25 mg resulted in a stronger decline of the fresh weight of granuloma and the incorporation of ^{35}S sulfate in the SMPS of connective tissue (Fig. 4).

FIGURE 4. ^{35}S Sulfate Incorporation Rate in the Tissue of Cotton Pellet Granuloma and the Fresh Wieght of Granuloma After 14-Day Treatment with 10 mg or 25 mg Vitamin E Per Day i. m. Implantation of Cotton Pellets on 9th Day After Start of Treatment, Investigation of Specific ^{35}S Sulfate Activity and Determination of the Fresh Weight of Granuloma 24 Hours After Last Vitamin E Application. Control Animals Were Not Treated.

Key: 1. Day; 2. Application; 3. Specific activity of ^{35}S-cpm/100 y SMPS in % of controls; 4. Fresh weight of granuloma; 5. Controls.



3.5. Inhibition of Non-Specific Mesenchyme Reaction to Toxin Stimulation by Vitamin E Pretreatment.

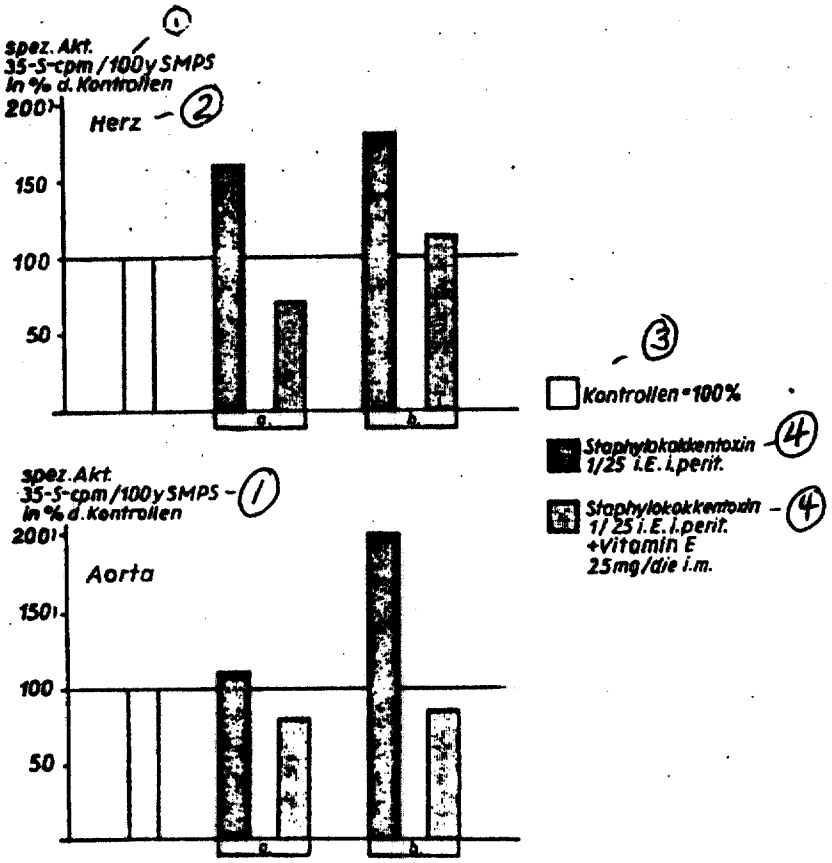
As shown by the authors earlier, an intraperitoneal toxin injection results in a non-specific mesenchyme reaction in the form of an increase of ^{35}S sulfur incorporation in the SMPS of the connective tissue of different organs. A Vit. E treatment before and during the effect of the toxin stimulation with a dosage of 25 mg Vit. E/day completely suppressed the reaction in the

connective tissue of the heart and the aorta. Both 24 and 48 hours after the toxin injection, a clear increase in the ^{35}S sulfate incorporation rates occurred in the SMPS of the connective tissue of untreated animals. The mesenchyme metabolic activity of animals pretreated with Vit. E, on the other hand, was less than the values of untreated control animals (Fig. 5).

FIGURE 5. Determination of the ^{35}S Sulfate Incorporation Rate in the Sulfated Mucopolysaccharides in the Heart and Aorta of Untreated Control Animals (=100%) After the Intraperitoneal Injection of 1/25 i. u. of Staphyloccous Toxin and After the Combined Administration of Vitamin E and Toxin:

a) 24 hours and
b) 48 hours after the staphyloccous toxin injection.

Key: 1. Specific activity of ^{35}S-cpm/100 y SMPS in % of controls; 2. Heart; 3. Controls; 4. Staphyloccous toxin.



4. Discussion

The results of the investigations showed:

1. A single Vit. E injection results in a prolonged inhibition of the intentisy of mesenchyme metabolism, particularly in the organs investigated.

2. The extend of this reaction of the connective tissue to the application of Vit. E is of different magnitude in different organs and is a function of dosage.

3. The inhibiting action of Vit. E on connective tissue metabolism can also be demonstrated under in-vitro experimental conditions.

4. The growth and the intensity of mesenchyme metabolism in cotton pellet granuloma are clearly reduced by pre- and simultaneous treatment with Vit. E.

5. The acceleration of metabolism triggered by toxin stimulation in the connective tissue of the heart and aorta is prevented by Vit. E treatment.

These results are in agreement with numerous clinical and animal experimental results of other authors: they demonstrate the importance of tocopherol in the regulation of connective tissue metabolism and the therapy of inflammatory, rheumatic and degenerative diseases localized in the mesenchyme /6, 11, 12, 35/.

Brown & al. /16/ showed that the synthesis of collagen in the skin of rate is reduced by the administration ov Vit. E, in comparision with animals maintained without tocopherol. According to Grifa /6/ and other researchers /5, 35/, Vit. E acts in vivo and in vitro as a hyaluronidase inhibitor and thus affects the structure of mesenchymal tissue, where hyaluronic acid possesses an important bonding function.

Other authors perceive in this process of hyaluronidase inhibition an explanation of the observation that Vit. E has a favorable effect on capillary resistance and permeability and on the formation of exsudative diathesis. /2, 4, 26, 27, 29/. Kannitz & al. showed in animal experiments that the triggering of a "Sanarelli-Shwartzman phenomenon" can be suppressed by Vit. E. /28/.

These processes, however, can also be explained by the authors experimental results. Based on information by Hauss and Jung-Hulsing concerning the structure and composition of the space esentially filled with connective tissue between the capillary and vessel walls and the parenchyme cell, the "transit route", the mesenchyme plays a decisive role for the function of the transport of electrolyte, nutritives and water in the organism. The control effect of Vit. E on mesenchyme metabolism explains the favorable

effect of tocopherol on all those inflamatory and degenerative processes which take place in the connective tissue and which in their sum play a decisive pathogenetic role in the causation of both rheumatic and arteriosclerotic diseases /18, 19, 20, 21, 25, 34, 39/.

The antiproliferative effect of Vit. E is clearly shown by the inhibition of the growth of granulation tissue of cotton pellet granuloma.

In addition, Vit. E, according to Hochrein & Zagga /22/ also has an influence on the intercellular water and sodium content; this places the heart muscle into a more favorable energetic position, which can be clinically demonstrated in the form of increased load-carrying capacity, especially of insufficient hearts, and of greater tolerance to strophantin. It is the opinion of the authors that this unambigously expresses the dehydrating, capacity increasing and toxicity reducing effect of tocopherol.

The authors findings show further that the reaction of the mesenchyme to toxin stimulation in the form of a substantial increase in metabolic activity can be suppressed by a Vit. E pretreatment of the animals. Noxa of different types, which include among others stress, nicotine, other toxic substances and also infections, generate accelerations of the connective tissue metabolism ("unspecific mesenchyme reaction"), which are additive (so-called addition phenomena) and finally lead to persistent, irreversible alterations of the mesenchymal tissue, clinically identified as arteriosclerosis, cardiac infarct, degenerative changes and premature aging processes. Hauss & al. /18, 19, 20, 21, 25, 34, 35/.

Based on experimental results, it therefore appears logical to provide an optimum supply of Vit. E, possibly even exceeding actual requirements, in the treatment of the pathological processes mentioned above.

Reductions were in the ^{35}S sulfate rate of incorporation in the SMPS of connective tissue were also demonstrated in vitro after Vit. E applications. This indicates that the inhibiting action of tocopherol on connective tissue metabolism, at least in part, is based on a direct effect on the regulating mechanisms of the mesenchyme, independently of endocrine or nervuos control processes. This is also supported by the fact that immediate on cell metabolism was found in tissue cultures, Beckman & Kuhlmann /5/.

In addition, effects of nucleic acid and amino acid metabolisms and the structure of intracellular orgella, such as mitochondrion and microsoma, of Vit. E were observed in tissue cultures. /11, 12, 29, 43/.

However, the probability that indirect effects of Vit. E may play a role in the numerous processes mentioned in the foregoing, is supported by the influence of tocopherol on the hormone balance, which was determined earlier /6, 26, 35, 40, 43/, the interaction with other vitamins /3/ and the enzyme metabolism /7, 37/. Presumably, this Vit. E effect is related to the importance of tocopherol as an antioxidant /3, 9, 29, 26, 38/.

The experimental results of the authors and numerous findings of others show that Vit. E has a function in the regulation of connective tissue, especially in connection with pathogenetic mechanisms, which by way of increasing the mesenchyme metabolism lead to damage to the structure and function of connective tissue and which clinically manifest themselves as inflammations, arteriosclerosis and syndromes from the rheumatic sphere of phenomena. It is characteristic that many of these diseases are accompanied by a reduced tocopherol content of the organism, also, that an increased consumption of Vit. E with rising age was found. The same appears to be true with respect to defense against infections and the healing of wounds /Beckman, 5/.

Summary

The effect of vitamin E on the connective tissue metabolism of rats was examined by means of the determination of ^{35}S-sulfate-installation rate in the SMPS of connective tissue. It could be seen that large vitamin E doses inhibit significantly the mesenchyme in vivo as well as in vitro.

Mesenchyme metabolism accelerations caused by toxin stimulation can be met with by large vitamin E doses, also the proliferation of the granulation tissue is diminished.

These findings confirm clinical and other experimental observations that vitamin E has a favourable effect in a great number of connective tissue diseases.

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Catabolism of Alpha-tocopherol in the Human Organism

by H. Schmandke[1]

Summary: In healthy male test subjects, the urinary excretion of tocopheronolactone was determined after different oral doses of dl-alpha-tocopherol. After the oral administration of 0.5, 1.0, 1.5, and 2.0 g of dl-alpha-tocopherol a total of 3.11±0.31 mg, 12.32±4.02 mg, 8.18±2.40 mg and 9.31±2.66 mg, respectively, of tocopheronolactone was excreted in the urine. The larger excretion of tocopheronolactone after the administration of 1.0 g dl-alpha-tocopherol in contrast to the excretion after 1.5 and 2.0 g can not be explained at present. The significance of these findings as an indication of the basal requirement of the human organism for alpha-tocopherol is discussed.

Alpha-tocopherol (I) is reduced to tocopheronolactone (II) in the human organism (8) and is excreted with the urine as a glucuronide (4). Alpha-tocopherol quinone (III) experiences the same conversion in the human organism (5). This shows that a catabolic pathway of alpha-tocopherol by way of alpha-tocopherol quinone to tocopheronolactone exists in the human organism (ring-splitting→ side-chain-shortening). Whether this conversion is of quantitative significance will be tested at this time in several test persons. The presentation of this question is authorized because in the rat organism 2,5,7,8-tetramethyl-2(beta-carboxyethyl)-6-hydroxychromane (IV) is reduced to tocopheronolactone by way of alpha-tocopherol quinone (1,2,6), and therefore in the rat a further catabolic pathway is possible (side-chain-shortening→ ring-splitting), which could also be present in the human organism.

In this work the dependence of the changes of the rate of catabolism of alpha-tocopherol to tocopheronolactone on the dose of alpha-tocopherol administered is imparted.

Results and Discussion

As is evident from Figure 1, the total excretion of tocopheronolactone in the urine of male test subjects after the oral administration of 0.5 g dl-alpha-tocopherol amounts to 3.11± 0.31 mg, after 1.0 g dl-alpha-tocopherol 12.32±4.02 mg, after 1.5 g dl-alpha-tocopherol 8.18±2.40 mg and after 2.0 g dl-alpha-tocopherol 9.31±2.66 mg. The fall in the tocopheronolactone excretion with an administered dose of 1.5 g compared with 1.0 g dl-alpha-tocopherol is significant ($p < 0.05$). This is not the case in the comparison of the diminished tocopheronolactone excretion after administration of 2.0 g in comparison with 1.0 g dl-alpha-tocopherol.

As a detailed analysis of the proportions, in Figure 2 the average daily excretion of tocopheronolactone in the urine following doses of different quantities of dl-alpha-tocopherol is given.

It is clear that the tendency present in Figure 1 for the total tocopheronolactone excretion to be in distinct quantities becomes apparent by the representation of the daily maximal, minimal, and average excretion.

In Figure 3 the course of the average daily excretion of tocopheronolactone for the individual administered doses of dl-alpha-tocopherol is represented. Hence it follows that for the individual doses, with increased excretion time the tocopheronolactone content in the urine of the test persons diminishes. In addition, Figure 3 shows that the excretion rate of tocopheronolactone per unit of time is highest after the administration of 1.0 g dl-alpha-tocopherol. This result is shown especially clearly in Figure 4. Here are the calculated regression lines represented, which also indicate the course of the average daily excretion of tocopheronolactone for the different doses of dl-alpha-tocopherol. The regression lines which correspond to the dl-alpha-tocopherol doses of 0.5, 1.5, and 2.0 g exhibit courses of the same kind as that for 1.0 g and at times differ significantly ($p < 0.002$).

It can not be explained at this time why there is a clearly higher urinary excretion rate of tocopheronolactone after the administration of 1.0 g dl-alpha-tocopherol than after administration of 1.5 and 2.0 g. Of the numerous possibilities - such as a change in the absorption, catabolism, or excretion rate; further catabolism of tocopheronolactone in the organism - at present the absorption rate in man under the same conditions is being worked on.

The significance of the rate of catabolism of alpha-tocopherol to tocopheronolactone should only be referred to here as a contribution to the basal requirement of man for alpha-tocopherol, which was limited by the absorption limit, measured at the average daily maximal excretion of 3.81±1.2 mg tocopheronolactone, amounted to 5.9 mg/day; the highest possible minimal catabolism of alpha-tocopherol, corresponding to the average daily minimal excretion of 0.53± 0.10 mg tocopheronolactone, was calculated at 0.8 mg/day[2].

1. I thank Dr. V. Erhardt for working the mathematical problems and G. Aust for technical assistance.
2. The molecular weights of tocopheronolactone and alpha-tocopherol are in the ratio of 1 : 1.55.

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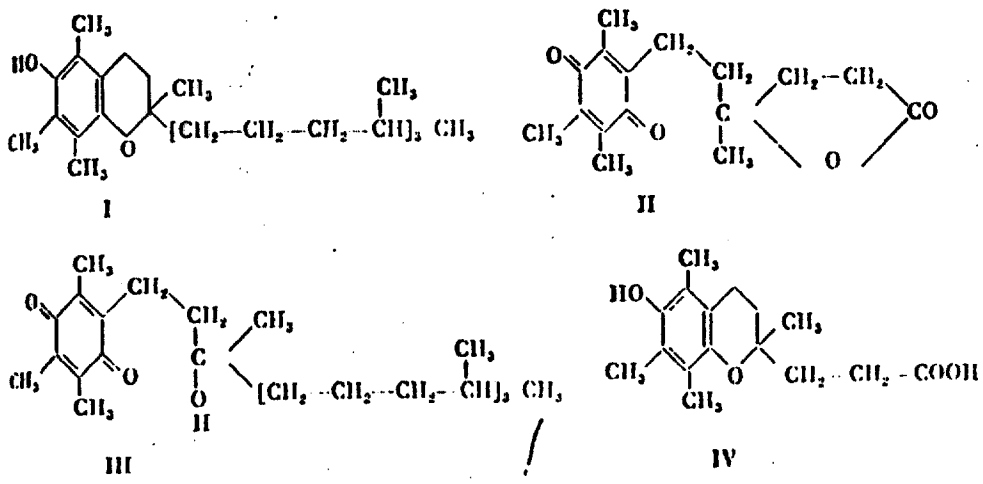

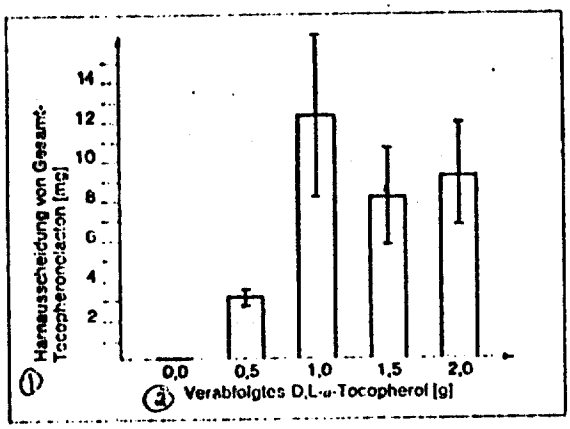

Figure 1. Schematic representation of the total excretion of tocopheronolactone in the urine of male test persons after the administration of various doses of dl-alpha-tocopherol.

Key: 1. Urinary excretion of total tocopheronolactone (mg)
2. Administered dl-alpha-tocopherol

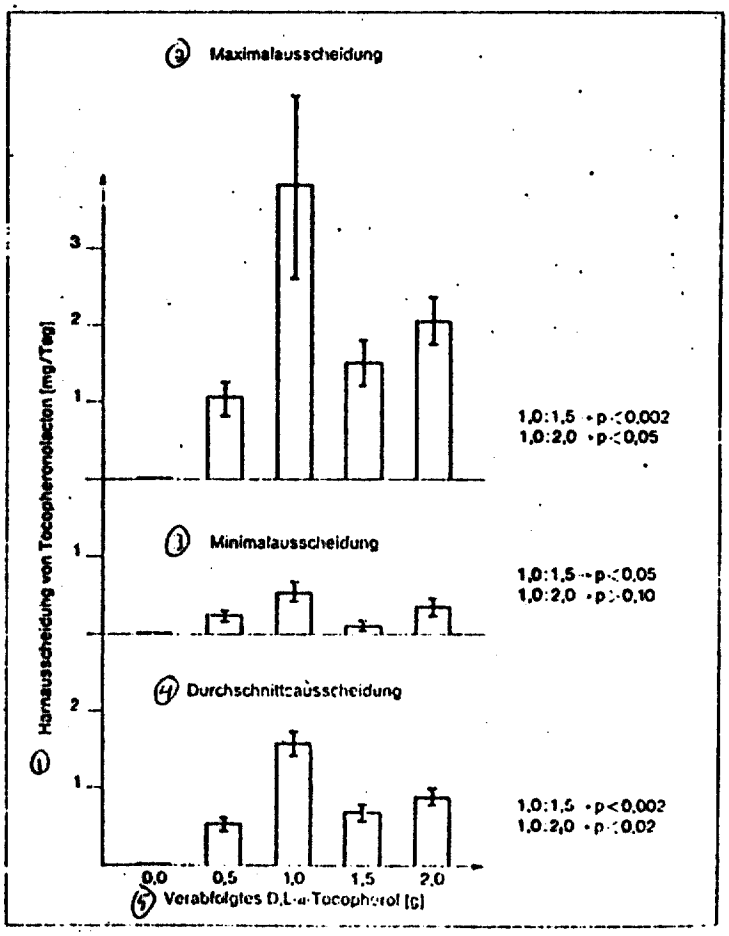

Figure 2. Schematic representation of the daily maximal, minimal, and average excretion of tocopheronolactone in the urine of male test persons after the administration of various quantities of dl-alpha-tocopherol.

Key: 1. Urinary excretion of tocopheronolactone (mg/day)
2. Maximal excretion
3. Minimal excretion
4. Average excretion
5. Administered dl-alpha-tocopherol (g)

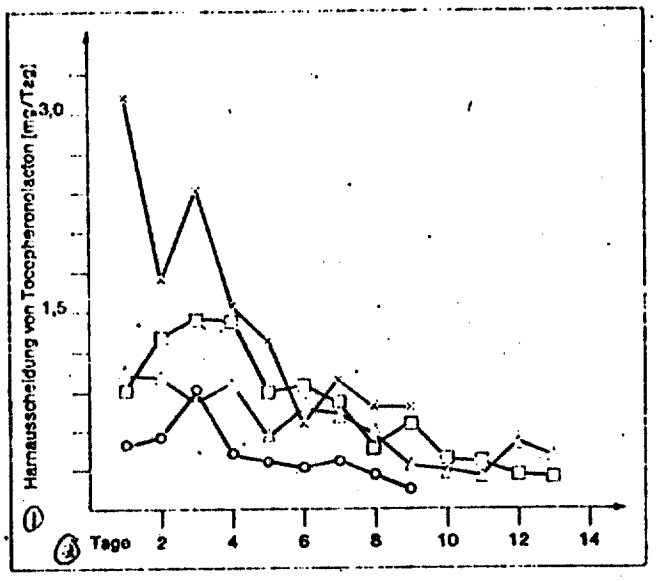

Figure 3. Course of the average daily excretion of tocopheronolactone in the urine of male test persons after the administration of various doses of dl-alpha-tocopherol.

(O—O 0.5 g; ×—× 1.0 g; Δ—Δ 1.5 g; □—□ 2.0 g).

Key: 1. Urinary excretion of tocopheronolactone (mg/day)
2. Days

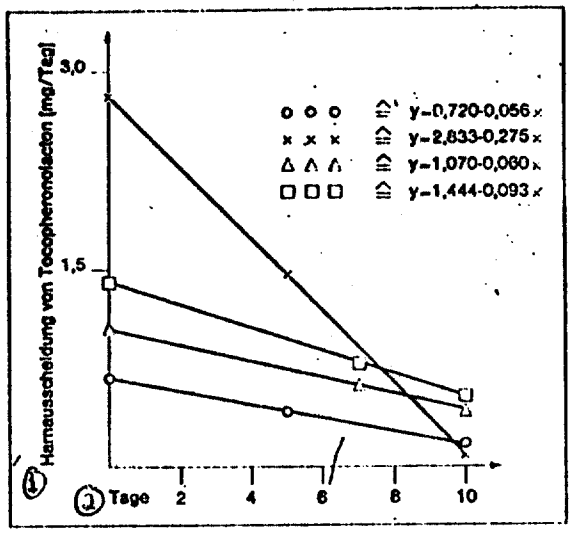

Figure 4. Course of the average daily excretion of tocopheronolactone, calculated with the help of the indicated regression equation, in the urine of male test persons after the administration of various doses of dl-alpha-tocopherol.

(O—O 0.5 g; ×--× 1.0 g; Δ--Δ 1.5 g; □--□ 2.0 g).

Key: 1. Urinary excretion of tocopheronolactone (mg/day)
2. Days

Int. Z. Vitaminforsch 38(1): 75-78 (1968)

Catabolism of Alpha-Tocopherol in the Human Organism

by H. Schmandke and G. Schmidt

Summary: After the peroral administration of 1 g dl-alpha-tocopherol to healthy male human subjects, 39.8±15.2% is absorbed and 6.0±2.4 mg dl-alpha-tocopherol/100 mg absorbed dl-alpha-tocopherol is reduced to tocopheronolactone; of 1 g of orally administered dl-alpha-tocoquinone 74.7±6.1% is absorbed and 1.8±1.4 mg dl-alpha-tocoquinone/100 mg absorbed dl-alpha-tocoquinone is reduced to tocopheronolactone. The low rate of change of dl-alpha-tocoquinone to tocopheronolactone in comparison with dl-alpha-tocopherol shows that the mechanism of alpha-tocopherol catabolism by way of alpha-tocoquinone to tocopheronolactone in the human organism plays only a small part in the catabolism of alpha-tocopherol to tocopheronolactone.

As earlier reported (4), we found in man that alpha-tocopherol can be reduced to tocopheronolactone mainly by way of alpha-tocoquinone. This finding in man was confirmed by Gloor et al. (2, 3) in so far as they demonstrated a reduction of alpha-tocoquinone to tocopheronolactone in the rat organism also. Gloor and Wiss (1) determined that after a dose of alpha-tocoquinone approximately double the quantity of tocopheronolactone was excreted in the urine of the rat as when alpha tocopherol acetate is administered, whereby they could verify that the absorption rates of alpha-tocopherol and alpha-tocoquinoe are very similar. That indicates that in the rat organism the reduction of alpha-tocopherol to tocopheronolactone proceeds to a high degree by way of alpha-tocoquinone.

In continuation of our preceding studies (5), under consideration of the absorption of alpha-tocopherol and alpha-tocoquinone, the quantitative reduction of both compounds to tocopheronolactone in thehuman organism shall be determined n order to obtain an indication of the catabolism of alpha-tocopherol in man.

Methods

Healthy male subjects from 31 to 38 years in age were each given 1 g dl-alpha-tocoquinone orally without solvent; tocopheronolactone was determined polarographically in the 24-hour-urine (6) and, after reduction with L-ascorbic acid, alpha-tocoquinone, as alpha-tocopherol, was determined by thin-layer chromatography in the feces (7). The reduction of the sample took 30 minutes at 70°C with 250 mg of L-ascorbic acid in 7 ml ethanol and 3 ml concentrated hydrochloric acid.

To the same test subjects, we administered, four weeks after conclusion of the tests with alpha-tocoquinone, 1 g dl-alpha-tocopherol orally, also without solvent. The determination of tocopheronolactone in the urine and of alpha-tocopherol in the feces was carried out as described above.

The absorption rate of the appropriate compounds resulted from the difference between the quantity administered and the quantity excreted in the feces.

The dl-alpha-tocoquinone was prepared and chromatographically

Table 1. Absorption of dl-alpha-tocopherol and dl-alpha-tocoquinone and their reduction to tocopheronolactone in the human organism.

① Versuchsperson	② Peroral Verabfolgung von 1 g D,L-α-Tocopherol				⑦ Peroral Verabfolgung von 1 g D,L-α-Tocochinon			
	③ Resorption von D,L-α-Tocopherol (%)	④ Tocopheronolacton-Ausscheidung im Harn (mg)	⑤ Abgebautes D,L-α-Tocopherol (mg)	⑥ bezogen auf resorbiertes D,L-α-Tocopherol (mg/100 mg)	⑧ Resorption von D,L-α-Tocochinon (%)	④ Tocopheronolacton-Ausscheidung im Harn (mg)	⑨ Abgebautes D,L-α-Tocochinon (mg)	⑩ bezogen auf resorbiertes D,L-α-Tocochinon (mg/100 mg)
1	51,2	11,9	18,4	3,6	78,1	17,6	27,3	3,5
2	26,1	12,7	19,7	7,5	79,8	15,1	23,4	2,9
3	60,1	12,5	19,4	3,2	69,4	7,3	11,3	1,6
4	33,2	15,8	24,5	7,4	66,8	4,5	7,0	1,0
5	28,6	15,3	23,7	8,3	79,2	1,2	1,9	0,2
$\bar{x}$	39,8			6,0	74,7			1,8
±	±			±	±			±
s_x	15,2			2,4	6,1			1,4

Key;
1. Test subject
2. Peroral administration of 1 g dl-alpha-tocopherol
3. Absorption of dl-alpha-tocopherol
4. Tocopheronolactone excretion in the urine
5. Reduced dl-alpha-tocopherol
6. Related to absorbed dl-alpha-tocopherol
7. Peroral administration of 1 g dl-alpha-tocoquinone
8. Absorption of dl-alpha-tocoquinone
9. Reduced dl-alpha-tocoquinone
10. Related to absorbed dl-alpha-tocoquinone

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The Absorption of Alpha-Tocopherol by Man

by H. Schmandke, C. Sima, and R. Maune

Since little is known of the absorption of alpha-tocopherol by man(1,4), the dependence of the absorption rate of alpha-tocopherol on the applied dose was determined in connection with our investigations on the metabolism of alpha-tocopherol in man.

Experimental

Thirty men between 18 and 34 years in age were used as test subjects, of whom 25 were nourished with the same community diet; the remaining 5 persons partook at lunch of the community food mentioned and obtained their breakfast and supper at home. Application of the DL-alpha-tocopherol (10,30,50,100,500,1500, and 2000 mg) was peroral, without solvent, on the fasting stomach. The consumption of breakfast followed at the earliest a half hour later.

In the 24-hour-excrement, alpha-tocopherol and, after reduction with L-ascorbic acid (HCl/ethanol (1:2), 70°C, 30 min), the alpha-tocopherol reduced to alpha-tocoquinone together with the unchanged alpha-tocopherol was determined by thin-layer chromatography, as alpha-tocopherol. The determination of alpha-tocopherol plus alpha-tocoquinone was carried out until the attainment of their normal excretion in the feces. The normal excretion of alpha-tocopherol and alpha-tocoquinone was determined at least once for each test subject.

Results

The average rates of absorption of DL-alpha-tocopherol following the pertinent, applied doses of DL-alpha-tocopherol are summarized in Table 1. The tendency is recognizable that with high doses of DL-alpha-tocopherol the absorption rate decreased. As a parallel it is quite well known to us that Klatskin and Molander (2) determined that with healthy men, 64% of theabsorbed quantity (calculated) of tocopherol is excreted with the feces. Rosenkrantz and coworkers (3) recovered, after the administration of 600 mg of alpha-tocopherol to one person, 50% in the feces. In individual tests we determined in male test subjects from 31 to 38 years of age, after the administration of 1 g DL-alpha-tocopherol, an absorption rate of 39.8±15.2 (6).

As further information it was imparted that our test subjects exhibited a normal daily excretion of 3.5±2.0 mg alpha-tocopherol plus alpha-tocoquinone and that after the administration of various quantities of DL-alpha-tocopherol, 50.6±23.6% was excreted with the 24-hour-feces as alpha-tocoquinone and the rest as alpha-tocopherol. It can not be judged how high the percentage of alpha-tocoquinone is at the absorption of alpha-tocopherol, in connection with which it is taken into consideration that in man alpha-tocoquinone is absorbed better than alpha-tocopherol.

Summary

After the peroral administration of 10,30,50,100,500,1500, and 2000 mg DL-alpha-tocopherol to male test subjects, the corresponding absorption rates amounted to 96.9±13.0%, 87.3±13.5%, 72.6±11.6%, 81.5±6.1%, 67.6±19.4%, 70.3±27.9% and 55.2±28.8%.

Table 1. Absorption of DL-alpha-tocopherol by man.

D.L.-α-Tokopherol-dose (mg)	Absorbed DL-alpha-toconherol									
	Test number								$\bar{x} \pm s$ (mg)	$\bar{x} \pm s$ (%)
	1 (mg)	2 (mg)	3 (mg)	4 (mg)	5 (mg)	6 (mg)	7 (mg)	8 (mg)		
10	9.7	8.2	7.8	10.0	10.0	11.5	10.6	–	9.7 ± 1.3	96.9 ± 13.0
30	22.8	28.1	30.2	20.4	28.5	25.8	31.0	22.2	26.1 ± 3.9	87.3 ± 13.5
50	39.1	27.3	35.5	35.5	46.9	37.8	31.1	36.5	36.2 ± 5.8	72.6 ± 11.6
100	90.9	88.7	80.5	74.7	76.4	–	78.5	81.0	81.5 ± 6.1	81.5 ± 6.1
500	408.0	281.1	321.9	482.4	446.7	231.8	231.4	295.3	337.3 ± 96.8	67.6 ± 19.4
1500	1398.0	475.0	1178.4	1003.2	1264.7	1266.8	1062.1	782.0	1053.8 ± 301.3	70.3 ± 27.9
2000	–	606.7	437.4	1368.4	808.9	–	1921.8	1480.6	1104.0 ± 576.1	55.2 ± 28.8

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THE EFFECT OF LARGE DOSES OF VITAMIN E ON MICROELEMENT METABOLISM IN AN ANIMAL ORGANISM

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In our earlier experiments, the presence of a correlation between vitamin E and the microelements in an animal organism was established. Thus, in experimental E-avitaminosis, the liver content of zinc, manganese, and cobalt is increased and the copper content decreased. In the muscles, manganese and cobalt content decreases. In the lungs, brain, and bones of E-avitaminosed rabbits, the amount of iron increases, whereas in the kidneys, it decreases.

According to data of O.Y. Voynar (3), the symptoms of hypovitaminosis E in animals are the same as for manganese deficiency in the organism. F. Ya. Berenshteyn affirms that addition of manganese salt to the ration of E-avitaminosed rats prevents E-avitaminosis. Ya. M. Berzin established that cobalt supplements influence the accumulation of vitamin E in organs and tissues.

Introduction to animals of surplus iron, in addition to causing its accumulation in organs, also results in a vitamin E deficiency, while the latter introduced supplementally interferes with iron accumulation in the organism, thus illustrating a defensive reaction.

The effect of surplus doses of vitamin E on microelement metabolism has not been sufficiently examined in current literature. According to data of some authors, it is evident that under the influence of vitamin E, molybdenum accumulates in the organism of animals.

Considering the fact that large doses of vitamin E are given during the treatment of some diseases, we decided to study the effect of large doses of this vitamin on iron, copper, and cobalt content in the organism of ani-

mals.

METHODS

Experiments were conducted on young albino rats weighing from 50-80 g. Forty animals, divided equally into four groups, were used. The rats were kept for 22 days on an E-avitaminosis ration consisting of: corn starch (61%), casein (18%), lard (5%), fish oil (2%), a saline mixture (4%), and dry brewer's yeast (0.5 g.per rat). A large amount of vitamin E was added to the ration: one group received daily 25 mg. vitamin E per rat, another 50 mg., and a third 100 mg. per rat. The control group were animals that received daily 3 mg. of vitamin E, in addition to the E-avitaminosis ration. The experiments were conducted in winter (December-January). At the end of the experiment, the rats were decapitated, and copper, iron and cobalt content was noted in the liver, muscles, and bones, according to the method of H.O. Babenko. The tested organs were weighed, placed in porcelain containers, dried and placed in an oven at 350-400 degree C temperatures, the succeeding hot working distilled with nitric acid occurring three times. Simultaneously, two livers were burned in as much as this organ gives little ash.

375 analyses were conducted. The results of the quantitative determination of microelements were expressed in milligram percentages in ash. The obtained data was statistically worked up according to I.A. Oyvin (9).

TEST RESULTS

The data shown in the table, illustrating the iron content in organs of experimental and control animals, confirm that in the control animals, the liver is richest in iron content (mean 1102.0$\pm$ 56.9 mg.%), less iron being found in muscles (143.0$\pm$ 5.3 mg.%), and least iron being found in bones (6.1$\pm$ 0.1 mg.% in ash).

TABLE 1. Iron, Copper, And Cobalt Content In The Liver, Muscles, And Bones Of Rats With Introduction Of Large Doses Of Vitamin E, In Mg. In Ash

	B Печінка				C М'язи				D Кістки			
	E Доза вітаміну E, мг				E Доза вітаміну E мг				E Доза вітаміну E мг			
A	Контроль	25	50	100	Контроль	25	50	100	Контроль	25	50	100
	F Залізо											
M	1102,0	377,7	549,0	1268,2	143,0	99,5	161,9	150,0	67,7	176,5	88,6	21,9
±m	56,9	66,6	59,5	13,1	5,3	3,2	6,3	3,1	5,6	5,0	4,7	1,4
σ	127,7	133,2	119,0	294,7	1,7	9,7	19,9	9,9	17,9	15,9	15,2	4,5
t	—	8,3	6,8	2,89	—	7,0	2,2	1,1	—	13,3	2,8	7,8
	G Мідь											
M	18,4	25,4	21,5	26,8	10,5	15,6	18,1	22,7	6,1	7,1	14,0	8,6
±m	0,7	0,9	0,5	0,3	0,5	0,4	0,3	0,3	0,1	0,2	0,1	0,5
σ	1,6	1,8	1,1	0,7	1,6	1,4	1,1	1,1	0,3	0,8	1,7	1,8
t	—	5,1	3,6	11,0	—	7,9	12,8	11,2	—	4,3	6,1	4,8
	H Кобальт											
M	0,86	0,57	1,37	1,23	0,73	1,26	0,64	1,05	0,24	0,63	1,64	0,85
±m	0,2	0,6	0,1	0,3	0,5	0,08	0,02	0,09	0,02	0,03	0,5	0,08
σ	0,53	1,34	0,23	0,73	0,15	0,25	0,07	0,3	0,08	0,13	0,34	0,26
t	—	0,4	2,2	1,01	—	5,7	0,1	4,3	—	10,1	2,5	7,3

A. Control
B. Liver
C. Muscles
D. Bones
E. Vitamin E dosage
F. Iron
G. Copper
H. Cobalt

Vitamin E, introduced in large doses to rats, substantially changes the iron content in the tested organs. In addition, changes in iron content in various organs, given the same dose of vitamin E, do not always have the same direction. Thus, while vitamin E in doses of 25-50 mg. leads to a decrease of iron content in the liver, whereas 100 mg. increases it, relative to the control animals, the opposite occurs in the bones. Vitamin E in 25-50 mg. doses increases its content, whereas 100 mg. decreases it relative to the control. In the muscles, as in the liver, a 25 mg. dose of

of the vitamin leads to a decrease in iron content, while a 100 mg. dose increases the amount of iron, relative to the control. The data determined for iron content in muscles of animals receiving 50 mg. of vitamin E daily is the opposite of the liver data: in the liver, this dose causes a decrease in iron content, whereas in muscles, it leads to an increase, relative to the control group.

Data denoting copper content in experimental and control animals receiving large doses of vitamin E confirm that the liver was the richest in copper (18.4±0.7 mg.% in ash), less being found in the muscles (10.5±0.5 mg.% in ash), and least being found in bones (mean 6.1±0.1 mg. in ash).

Vitamin E, administered in large doses (25, 50, 100 mg.), leads to an increase in copper concentration in all tested organs. In addition, this increase has a similar character: in the liver, with introduction of 25 mg. of the vitamin, copper content increases by a greater measure than with introduction of 50 mg., while its introduction in 100 mg. doses leads to an even greater copper content; in bones 50 mg. increases copper content, while 100 mg. increases it even more in comparison with a 25 mg. dose. Copper content in muscles increases with increased vitamin E dosage.

In this manner, quantitative changes of copper content in tested organs and tissues have the same direction with introduction of large doses of vitamin E.

Data denoting cobalt content in organs of experimental and control animals shown in the table confirm the fact that in control animals, the liver is richest in cobalt (0.86±0.2 mg.% in ash), less being found in the muscles (mean 0.73±0.5 mg.% in ash), and least being found in bones (mean 0.24±0.02 mg.% in ash).

With introduction of large doses of vitamin E, cobalt content in the liver, muscles, and bones changes. It should be noted that the character of these changes are analogous to changes in copper content, that is, an increase in microelement content in organs and tissues of experimental animals.

The exception is in cobalt content of the liver in animals receiving 25 mg. of vitamin E and in the muscles of animals receiving 50 mg. However, these data were statistically inconclusive.

Analyzing the iron, copper, and cobalt content of the liver, muscles and bones with introduction of various doses of vitamin E, it is possible to note that in the tested organs and tissues, the content of all microelements is increased with the exception of iron. Iron content in the liver decreases with introduction of vitamin E in doses from 25-50 mg; in muscles with introduction of 25 mg., and in bones with introduction of 100 mg. vitamin E.

CONCLUSIONS

1. Oral introduction of 25, 50, and 100 mg. vitamin E to rats results in an increase of copper and cobalt concentrations in the liver, muscles, and bones.

2. Changes in iron content in organs and tissues of rats with introduction of various doses of vitamin E have a diverse character, specifically: (a) vitamin E in 25 mg. doses leads to a decrease of iron content in the liver and muscles, and to an increase in the bones; (b) 50 mg. of vitamin E leads to a decrease of an equal iron content in the liver and increase in muscles and bones; (c) 100 mg. of the vitamin increases iron content in muscles and liver, and decreses it in bones.

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Protection Against Cocarcinogenesis by Antioxidants

The metabolic sequence of events responsible for the initiation and promoting stages of co-carcinogenesis has not been elucidated.

The reactions of the co-carcinogens with skin components may produce free radicals or peroxidize lipid membranes. A hydroperoxide derived from cholesterol has been shown to be carcinogenic under certain conditions[1]. The main reaction of polycyclic hydrocarbons appears to be oxidation by hydrogen peroxide or an oxygen atom to activate double bonds, to yield intermediary epoxides[2].

Peroxidation may damage cells in several ways[3]: destruction of enzymes and cytochromes of electron transport; destruction of cytochrome b_5; breaking of lysosomal and microsomal lipid membranes and releasing of hydrolytic enzymes; and hemolysis of erythrocytes.

In this preliminary study, mice were first initiated with 7,12-dimethylbenzanthracene. Antioxidants and free radical inhibitors were applied concomitantly with the tumor promoter, i.e. croton oil.

30 ICR Swiss female mice, 55–60 days old, were initiated once with 125 γ 7,12-dimethylbenzanthracene dissolved in 0.25 ml acetone. After a period of three weeks, the animals were painted five times weekly for 16 weeks with 0.25 ml of a mixture of 0.033% croton oil and the various test substances dissolved in an 80% acetone, 20% water solution. The test substances were 0.0005% sodium selenide, 0.01% hydrocortisone, 0.25% D,L-α-tocopherol and 0.25% cysteamide. The group which received the croton oil alone was the positive control. One group received 7,12-dimethylbenzanthracene only. The animals were examined weekly and the number and distribution of tumors were noted. Results after 16 weeks are indicated in the Table.

Sodium selenide applied concomitantly with the croton oil markedly reduced tumor formation (Table). Selenium is a known powerful antioxidant[3]. Hydrocortisone and D,L-α-tocopherol also reduced tumor formation. Both substances protect against lipid peroxidation[5,6]. Cysteamide, known to protect against radiation[7], had a small protective effect. The relative effectiveness of each antioxidant was calculated (Table). Sodium selenide was 2780/3.78 or 735 times as effective as D,L-α-tocopherol against tumor formation. Selenium antioxidants on a molar basis are known to have 500 to 2000 times the antioxidant activity of D,L-α-tocopherol in vitro[3].

Antioxidants are likely to inhibit early critical oxidation and reduction reactions necessary for tumor formation[8].

Zusammenfassung. Natrium-Selenid und andere Antioxydantien können die Entstehung von Tumoren hemmen. Die Wirkung kommt möglicherweise zustande über eine Hemmung der Bildung von Co-Carcinogenen.

R. J. SHAMBERGER and G. RUDOLPH

Roswell Park Memorial Institute, Buffalo (New York USA), October 4, 1965.

The effect of antioxidants on the prevention of croton oil induced tumors

0.033% croton oil + addition	No. of tumors	Relative effectiveness[4]
Sodium selenide	9	2780.0
D,L-α-Tocopherol acetate	50	3.8
Hydrocortisone	55	65.8
Cysteamide	84	0.37
None	132	0
Dmba only	0	–

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THE EFFECT OF VITAMIN E GREAT DOSES ON MICROELEMENT METABOLISM IN AN ANIMAL ORGANISM

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Summary

The content of copper and cobalt in the liver, muscles and bones of animals is shown to increase at every-day administration to rats of vitamin E in great doses (25, 50 and 100 mg) for 22 days.

Changes in the iron content in the organs of rat tissues are in a definite dependence on the administrated dose of vitamin E: 25 mg of the vitamin results in a decrease of the iron content in the liver and muscles and in its increase in the bones; 50 mg of it causes a decrease in the iron amount in the liver and its increase in the muscles and bones; 100 mg of the vitamin decreases the iron content in bones and increases its concentration in the muscles and liver.

THE METABOLISM OF VITAMIN E

I. THE ABSORPTION AND EXCRETION OF *d*-α-TOCOPHERYL-5-METHYL-C^{14}-SUCCINATE*

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Vitamin E is widely distributed throughout the tissues of animals and man (2–4), and its deficiency causes a variety of syndromes in laboratory and farm animals (5). Very little information, however, is available concerning either the metabolic fate or the biochemical rôle of this vitamin. We have, therefore, undertaken a study of the metabolism of α-tocopherol.

It has been reported that α-tocopherol and α-tocopherylquinone were not excreted in the urine of animals even after the administration of large doses of vitamin E (6, 7). Some excretion of absorbed tocopherol into the intestinal tract has been postulated (7, 8). In this report we shall present definitive evidence both for the presence of a metabolic product of α-tocopherol in the urine and for the excretion of α-tocopherol and its metabolic products from the blood stream into the intestinal tract.

Materials and Methods

d-α-Tocopheryl-5-methyl-C^{14}-succinate—The crystalline succinate ester of *d*-α-tocopherol labeled with C^{14} in the 5-methyl group was used in these studies.[1] The material had a specific activity of 0.147 μc. per mg.

β-D-Glucuronidase—Beef spleen and rat liver preparations of β-D-glucuronidase (9) were used.[2]

Administration and Collection—Young rabbits (1 to 2 kilos) maintained on Diet 11 of Goettsch and Pappenheimer (10) supplemented with *dl*-α-tocopherol were kept in metabolism cages which permitted separation and

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A preliminary report on this work was presented before the 44th meeting of the American Society for Pharmacology and Experimental Therapeutics at Atlantic City, April, 1954 (1).

[1] The authors wish to thank Dr. P. L. Harris of Distillation Products Industries for making available the labeled *d*-α-tocopheryl succinate.

[2] The authors are indebted to Dr. M. Levitz of New York University College of Medicine, and Dr. D. Dziewiatkowski of The Rockefeller Institute for Medical Research for generous quantities of β-*d*-glucuronidase preparations.

complete collections of all feces and urine eliminated during each 24 hour period. The *d*-α-tocopheryl-5-methyl-C^{14}-succinate in doses of 10 to 15 mg. (1.5 to 2 μc.) was administered by various routes. The material was dissolved in 1 ml. of sesame oil for oral and subcutaneous administration. For intravenous injection it was dissolved in 1 ml. of a mixture of 9 parts of 5 per cent aqueous triethanolamine and 1 part of ethanol. Massive doses were administered by mixing 200 mg. of unlabeled *d*-α-tocopheryl succinate with the tracer dose in 6 ml. of aqueous triethanolamine-ethanol.

Extraction of Radioactive Materials—Urines were acidified to pH 1 to 2 and extracted continuously with ether for 48 hours. About 90 per cent of the radioactivity was recovered in this manner. Longer extraction times did not result in an increased yield of C^{14}. Feces were dried in vacuum and ground to a fine powder. Continuous extraction with ethanol in a Soxhlet apparatus for 24 hours yielded 80 to 90 per cent of the radioactivity. The yields were not improved by increasing the time of extraction.

Methods of Counting—Whenever levels of activity permitted, samples to be counted were converted to barium carbonate by a modification (11) of the wet combustion method of Van Slyke and Folch (12) and counted at "infinite thickness" in stainless steel planchets in a Tracerlab windowless gas flow counter. The factor relating counts per minute of "infinitely thick" barium carbonate to microcuries was determined by counting as barium carbonate a sample of *d*-α-tocopheryl-5-methyl-C^{14}-succinate of known weight and specific activity.

When combustion was not feasible, small urine aliquots were dried slowly in tared planchets under an infra-red lamp and counted. A self-absorption curve for these samples was constructed by counting a known quantity of *d*-α-tocopheryl-5-methyl-C^{14}-succinate with various quantities of urine residues. Samples of dried feces were counted directly at "infinite thickness." Conversion of counts per minute at "infinite thickness" to microcuries was accomplished by counting fecal samples of sufficiently high specific activity both directly and as barium carbonate. Agreement between counts by direct plating and combustion was reasonably good (±10 per cent).

Hydrolysis of Urinary Excretion Product; Acid Hydrolysis—To the residue of an evaporated ether extract of a 24 hour specimen of urine 100 ml. of 3 N HCl were added, and the mixture was refluxed for 2 hours. On cooling to room temperature the hydrolysis mixture was extracted with petroleum ether for 24 hours.

Enzyme Hydrolysis—The residue of an evaporated ether extract of a 24 hour specimen of urine was dissolved in 40 ml. of water, the pH was adjusted to 4.5 with solid sodium bicarbonate, and 4 ml. of acetate buffer (pH 4.5) were added. The mixture was incubated at 37° for 5 days with β-D-glu-

curonidase in concentration of 400 Fishman units per ml. Penicillin and Streptomycin, in concentrations of 25 units and 25 γ per ml., respectively, were added to prevent bacterial growth. The incubation mixture was subsequently extracted with petroleum ether for 24 hours.

Ultraviolet Spectra—Ultraviolet spectra were determined in purified ethanol (distilled over potassium permanganate and potassium hydroxide) and "spectrograde" isooctane (Phillips Petroleum) in silica cells with a 1 cm. light path in a Beckman DU spectrophotometer.

Paper Chromatography—Two reverse phase paper chromatographic systems, developed for the separation of the tocopherols, were employed: the system of F. Brown (13) as modified by Eggitt and Ward (14) in which Whatman No. 1 paper is impregnated with light petroleum, B. P., Nujol, and the chromatogram is developed with 75 per cent ethanol, and the system of J. A. Brown (15) in which Whatman No. 1 paper is treated with silicone stop-cock grease and developed with appropriate mixtures of acetonitrile and water. Spots were identified by scanning under an ultraviolet lamp and, in the case of α-tocopherol, by spraying with a ferric chloride-dipyridyl reagent in glacial acetic acid (16).

Isotope Dilution Technique—Known quantities of unlabeled *d*-α-tocopherol or *d*-α-tocopheryl succinate were added to extracts of feces or urine, and isolated as the crystalline succinate ester. In instances when free α-tocopherol had been added, the succinate was prepared by refluxing the dry residue with an excess of succinic anhydride in a few ml. of pyridine for 3 hours. Ether was added to precipitate the excess anhydride, which was removed by filtration. Repeated washings with 5 per cent HCl removed the pyridine. Ether was removed by evaporation. Isolation of tocopheryl succinate was achieved in the following manner: the mixture was dissolved in 83 per cent ethanol and made slightly alkaline. Extraction with three portions of petroleum ether removed impurities. After acidification of the ethanol solution, the tocopheryl succinate was extracted into petroleum ether. The crude tocopheryl succinate was recrystallized to constant specific activity from isooctane or petroleum ether. The total radioactivity of the tocopheryl succinate (specific activity times theoretical yield) divided by the total radioactivity in the extract represents the fraction of isotopic material present in the extract as tocopherol or tocopheryl succinate.

Isolation of Fecal Metabolite—A crude fecal metabolite was isolated by a modification of the series of steps described by F. Brown (13). *p*-Acetylaminophenol (17) was found to be a better antioxidant than pyrogallol during the saponification. In the chromatographic step α-tocopherol was eluted from Florex XXS with benzene; however, ether-ethanol mixtures were necessary to elute the metabolite.

RESULTS AND DISCUSSION

Absorption and Excretion Studies—The oral administration of a sesame oil solution of *d*-α-tocopheryl-5-methyl-C^{14}-succinate led to a rapid elimination of radioactivity in the feces, as is shown in Fig. 1. Over 74 per cent of the administered dose appeared in the stool within 3 days after administration. More than 93 per cent of the fecal excretion product was shown by the isotope dilution technique to be either α-tocopherol or α-tocopheryl succinate. Only traces of radioactivity were found in the urine.

The subcutaneous administration of labeled α-tocopheryl succinate in oil was followed by very slow elimination of radioactivity (Fig. 2). During

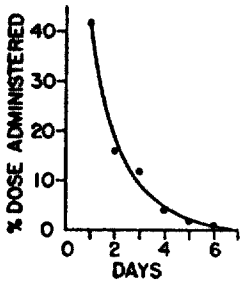

FIG. 1

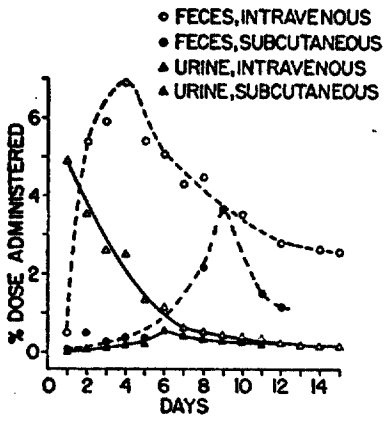

FIG. 2

FIG. 1. Excretion of radioactivity in feces after oral administration of a tracer dose of *d*-α-tocopheryl-5-methyl-C^{14}-succinate.

FIG. 2. Excretion patterns after subcutaneous and intravenous administration of tracer doses of *d*-α-tocopheryl-5-methyl-C^{14}-succinate.

11 days only 10 per cent of the dose appeared in the feces and 4 per cent in the urine. When the animal was sacrificed on the 11th day after injection, 14 per cent of the administered dose was found at the site of injection.

When the isotopic vitamin E was given by the intravenous route, considerable radioactivity was eliminated in both urine and feces, as shown in Fig. 2. The urinary activity was highest on the 1st day and decreased rapidly thereafter, while the fecal output reached its peak on the 3rd or 4th day after injection and fell off more slowly. A period of 15 to 20 days was required for complete elimination of the isotopic material as shown by the absence of detectable radioactivity from both urine and feces after this period. The total recovery of C^{14} from the excreta comprised 70 to 75 per cent of the administered dose. About 20 to 30 per cent of the recovered radioactivity appeared in the urine, while 70 to 80 per cent was found in the feces.

When a "massive dose" of tocopheryl succinate was administered by the intravenous route, the total amounts of radioactivity appearing daily in the excreta were roughly the same as when only the tracer dose was given. This would seem to indicate that the fraction of the dose that is eliminated by the body is essentially independent of the size of the dose in the range of 10 to 220 mg.

Isotope dilution studies showed that in the feces of rabbits given α-tocopheryl succinate either subcutaneously or intravenously 40 to 50 per cent of the ethanol-extractable excretion products was free α-tocopherol. Virtually all of the succinate ester had been hydrolyzed in the body.

Previous reports on the absorption of vitamin E are few and are limited largely to studies of absorption from the gastrointestinal tract (18, 19). The results were usually based on the chemical determination of tocopherol and tocopherylquinone in the feces and are somewhat open to question in view of the possibility of excretion of absorbed tocopherol into the intestinal tract. The use of isotopic tocopherol permits the estimation of absorption by comparison of the rate of fecal excretion, the level of urinary radioactivity, and the nature of the fecal excretion products with the corresponding results obtained on intravenous administration of the material. Such a comparison leads to the conclusion that most of the labeled material recovered in the feces after oral administration, particularly during the first 3 days, must have been unabsorbed vitamin E, since after intravenous injection only 12 per cent of the dose had appeared in the stool during the same period, and since only 40 to 50 per cent of this material was α-tocopherol. A consideration of these results and of the relative levels of urinary radioactivity led us to estimate that the amount of α-tocopheryl succinate absorbed from the gastrointestinal tract in this experiment did not exceed 10 per cent of the oral dose.

It is of interest that following subcutaneous administration the excretion patterns, including the relative amounts of radioactivity in urine and feces, resemble closely those obtained on intravenous injection, except for smaller absolute amounts of daily excretion and a delay of both maxima by 6 days, presumably due to the very slow rate of absorption from the subcutaneous site. Since the amount of tocopherol absorbed averaged approximately 1 mg. per day, or about the quantity an animal may be expected to absorb from its food, this may be taken as evidence that these excretion patterns would hold under physiological conditions.

A direct investigation of radioactivity present in the respiratory carbon dioxide was not feasible because of the low level of radioactivity of the administered material. It seems justified to conclude, in view of the high recovery of radioactivity in urine and feces over such a long period, that oxidation of the carbon atom of the 5-methyl group and its excretion as carbon dioxide can at best be a very minor pathway of elimination.

The data presented indicate that a significant portion of parenterally administered tocopherol is eliminated in some form in the urine and that excretion from the blood stream into the intestinal tract takes place to a large degree. These results bear considerable similarity to the findings of Siperstein and Chaikoff (20) on the elimination by the rat of intravenously administered C^{14}-cholesterol labeled in the steroid nucleus. These authors found that virtually all of the isotopic cholesterol was excreted into the intestinal tract and eliminated in the feces. The period of 15 days required for complete elimination of the administered dose as well as the shape of the fecal excretion curve is strikingly similar to the results reported here for vitamin E. The observation that elimination via the urine is a significant pathway for α-tocopherol, while it is a negligible one for cholesterol, represents the most important difference between the modes of elimination of these two lipide materials.

Nature of Excretion Products Following Parenteral Administration: In Feces—It has already been pointed out that 40 to 50 per cent of the ethanol-extractable fecal excretion products was found to be free α-tocopherol. Preliminary observations on the balance of the fecal radioactivity indicate that it is composed of materials of much more polar character than α-tocopherol, as shown by its distribution between aqueous ethanol and petroleum ether and the behavior of a crude metabolite, isolated from the feces, in reversed phase paper chromatography. This material had an R_F of 0.88 in the system of Eggitt and Ward (14), in which the R_F of α-tocopherol is 0.25. The nature of the fecal excretion products is under investigation.

In Urine—The reason for the negative findings of previous workers looking for urinary α-tocopherol became clear when it was shown by isotope dilution as well as by paper chromatographic procedures that no detectable α-tocopherol or α-tocopheryl succinate was present in urine. On the assumption that small quantities of tocopherol, if they were to appear in the urine, would most likely be present early after administration, when the blood α-tocopheryl succinate level is highest, the urine of one rabbit was collected by catheter during the 6 hours immediately following intravenous injection. The first urine sample obtained 2 hours after injection had the highest specific activity, but, even here, metabolic products of α-tocopherol accounted for 99 per cent of the radioactivity.

Over 90 per cent of the isotopic material in the urine was strongly acidic and could be removed from acidified urine by continuous ether extraction for 48 hours. Continuous extraction with petroleum ether removed less than 10 per cent of the total radioactivity.

Acid hydrolysis of the evaporated ether extracts increased the amount of petroleum ether-extractable isotopic material to 60 to 70 per cent, while incubation with the enzyme β-D-glucuronidase gave a petroleum ether

soluble radioactive substance in 70 to 90 per cent yield. These results provided evidence that the major excretion product may be a glucuronide.

Since hydrolysis and petroleum ether extraction gave a considerably purified product, as indicated by its spectrum and specific activity, our attention was directed towards a study of this material. Urines of rabbits injected intravenously with massive doses of *d*-α-tocopheryl succinate were used. The petroleum ether extracts of acid or enzyme hydrolysates from the urines of such rabbits showed the characteristic absorption spectrum depicted in Fig. 3. The proportionality of the optical density at the max-

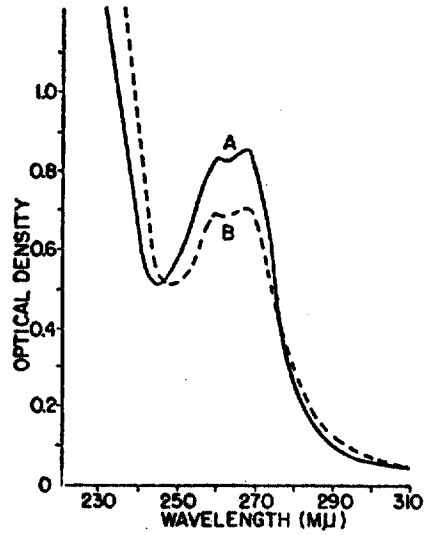

FIG. 3. Ultraviolet absorption spectra of urinary metabolite after hydrolysis and petroleum ether extraction. Curve A, after enzyme hydrolysis; Curve B, after acid hydrolysis.

ima to the specific activity of the sample as well as the similarity of the spectrum to that of α-tocopherylquinone (21), from which it differs only by the presence of end-absorption in the short ultraviolet region, was taken as evidence that the spectrum is really due to the metabolite rather than to impurities. The substance was shown not to be α-tocopherylquinone by its considerably more polar character. While the latter is quantitatively extracted from 50 per cent ethanol by one extraction with an equal volume of petroleum ether, the metabolite distributes itself between these solvents at a ratio of 3:1 in favor of the aqueous ethanol. In the chromatographic system of Eggitt and Ward (14) the metabolite had an R_F of 0.88 compared to 0.50 for α-tocopherylquinone and 0.25 for α-tocopherol. An even more striking demonstration of the vast difference in polarity is shown in Table I,

which gives the R_F values for these compounds in the system of J. A. Brown (15).

Whereas tocopherol and its quinone separated only slightly in this system, the metabolite traveled with the solvent front in concentrations of aqueous acetonitrile in which tocopherol and its quinone did not leave the origin.

Paper chromatography of the hydrolyzed material also indicated the complete absence of a conjugated form of α-tocopherol.

The increase in polarity and the characteristic spectrum of the urinary metabolite obtained on hydrolysis point to oxidation of the chroman ring to the *p*-quinone state and to drastic reduction and possible alteration of the 16-carbon isoprenoid side chain of α-tocopherol. Further purification

TABLE I

Paper Chromatography of α-Tocopherol, α-Tocopherylquinone, and Hydrolyzed Urinary Metabolite of Vitamin E

Compound	R_F value					
	Per cent acetonitrile in developing solvent					
	20	30	40	50	70	90
α-Tocopherol	0	0	0	0	0.29	0.75
α-Tocopherylquinone	0	0	0	0	0.35	0.82
Metabolite	0.60	0.74	0.83	S. F.*	S. F.	S. F.

Whatman No. 1 filter paper impregnated with silicone grease (14) was developed by the ascending technique with the concentrations of aqueous acetonitrile indicated.

* Solvent front.

and characterization of this compound had to await the discovery of a more lucrative source which permitted the accumulation of mg. quantities of the material. This work will be discussed in Paper II of this series (22).

SUMMARY

1. *d*-α-Tocopheryl-5-methyl-C^{14}-succinate was administered to young rabbits by various routes, and its absorption and excretion were studied.

2. Absorption from the gastrointestinal tract and from the subcutaneous site was low when an oily vehicle was used.

3. Complete excretion of radioactivity after intravenous administration required 15 to 20 days. Urine contained 20 to 30 per cent and feces 70 to 80 per cent of the radioactivity recovered from the excreta.

4. The excretion pattern after subcutaneous injection resembled that obtained after intravenous administration, except for a delay of the maximum by 6 days.

5. Free α-tocopherol was found to comprise 40 to 50 per cent of the ethanol-extractable fecal radioactivity after parenteral administration of the tracer dose.

6. The bulk of the urinary radioactivity was shown to be a metabolic product of α-tocopherol. Evidence is presented that it may be conjugated with glucuronic acid. Some of its properties are discussed.

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THE METABOLISM OF VITAMIN E

II. PURIFICATION AND CHARACTERIZATION OF URINARY METABOLITES OF α-TOCOPHEROL*

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Studies with *d*-α-tocopheryl-5-methyl-C^{14}-succinate in rabbits demonstrated the presence of a conjugated metabolite of vitamin E in the urine (2). The product obtained on enzyme or acid hydrolysis, as well as the small amount of unconjugated metabolite excreted, had an ultraviolet absorption spectrum similar to that of α-tocopherylquinone. It was shown, however, to be considerably more polar in nature than the latter, suggesting that rather profound alterations in the structure of α-tocopherol may have taken place.

Urines of humans ingesting large doses of *dl*-α-tocopherol were found to be a good source of a conjugated substance, which on hydrolysis showed spectroscopic, chromatographic, extraction and chemical properties identical with those of the material obtained from rabbit urine. More careful study revealed that two metabolites, one neutral and the other acidic, but both with the same characteristic ultraviolet absorption spectrum (Fig. 1), were obtained on hydrolysis of human urine.[1] The purification and chemical characterization of these metabolites as 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone and its γ-lactone will be described. Neither of the metabolites nor the crystalline derivative of the lactone, the preparation and properties of which will be described, has previously been reported in the literature. These structures should, therefore, be considered tentative until definitive proof has been obtained by synthesis.

* This work was supported by Muscular Dystrophy Associations of America, Inc., and by assistance from the Tall Cedars of Lebanon. A preliminary report of this work was presented at the 46th meeting of the American Society of Biological Chemists at San Francisco, April, 1955 (1).

[1] The amount of acidic metabolite present in rabbit urine was so small that it originally escaped notice. It has since, however, been found to be present there as well.

EXPERIMENTAL

Subjects and Administration—The subjects were persons who were either free from any known disease or had evidence of muscular wasting. During the periods of investigation the subjects were given daily oral doses of 3 to 5 gm. of *dl*-α-tocopherol in gelatine capsules. The urine was collected under careful supervision in 24 hour samples.

Ultraviolet Spectra—Ultraviolet absorption spectra were determined in ethanol purified by distillation over solid potassium hydroxide and potassium permanganate, in silica cuvettes with a 1 cm. light path in a Beckman DU spectrophotometer.

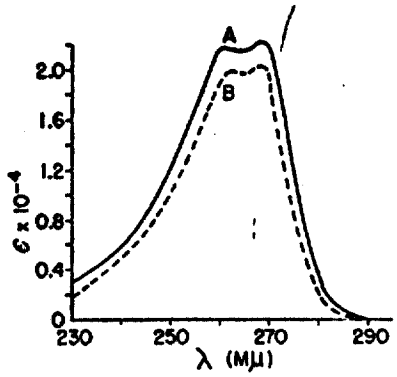


FIG. 1. Ultraviolet absorption spectra of the purified metabolites (Curve A) and of α-tocopherylquinone (Curve B).

Infra-Red Spectra—The infra-red spectra were obtained on a Perkin-Elmer model 21 double beam spectrophotometer. Solvents and prisms used are indicated in Fig. 2.[2]

Isolation and Purification—Urines (24 hour collections) were acidified to pH 1 and extracted continuously with ether for 48 hours. By the procedures described previously for rabbit urine (2) residues from pooled ether extracts were hydrolyzed with β-D-glucuronidase (Warner and Chilcote "ketodase"), and the hydrolysis mixtures were extracted with petroleum ether. Considerable hydrolysis could also be accomplished by letting the wet ether extracts stand at room temperature for several days. Approximately 15 to 30 mg. of crude metabolites per 24 hour urine specimen were obtained in the petroleum ether extracts. This mixture was separated into neutral and acidic fractions by extraction of the solution with aqueous so-

[2] The authors are indebted to the Division of Steroid Metabolism at the Sloan-Kettering Institute for Cancer Research for carrying out the infra-red analyses, and to Miss Friederike Herling and Dr. Glyn Roberts for valuable help in the interpretation of the spectra.

dium bicarbonate. The major portion of material remained in the petroleum ether. The acidic material was recovered by ether extraction of the acidified aqueous solution.

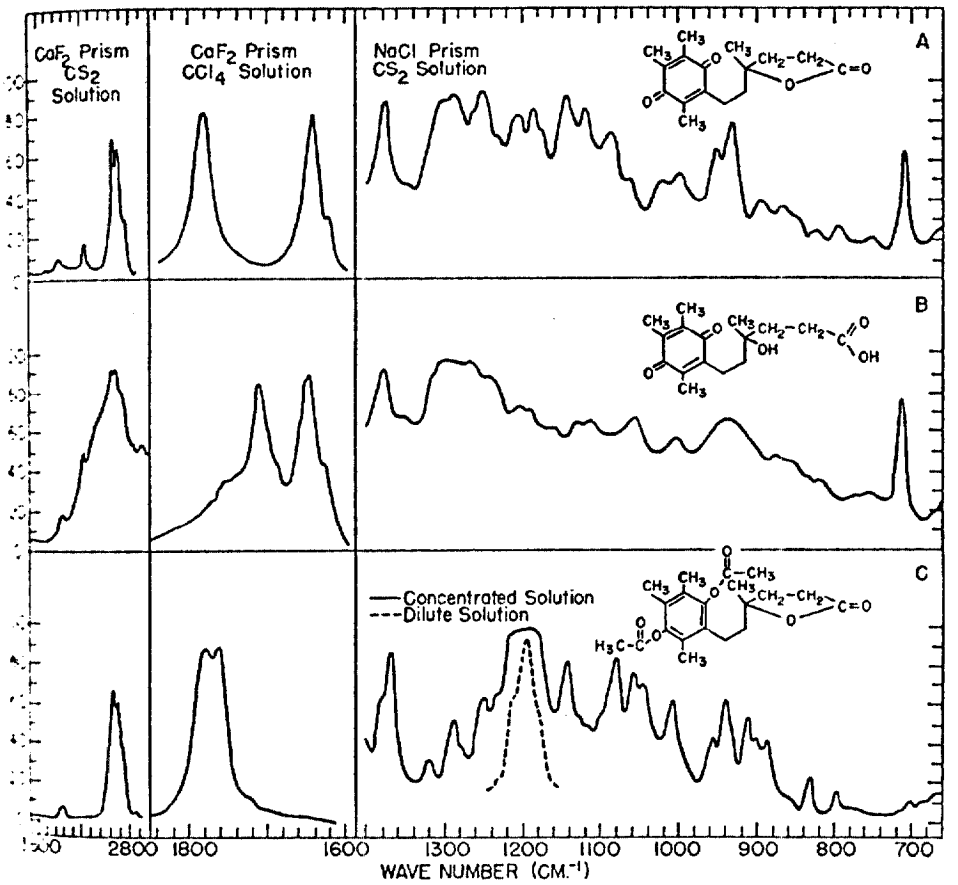


FIG. 2. Infra-red spectra of the metabolites, lactone (A) and hydroxy acid (B), and of the hydroquinone diacetate derivative of the lactone (C) from 4000 to 650 cm.$^{-1}$.

To avoid the acidification and ether extraction steps, enzyme hydrolysis of fresh urine, adjusted to pH 4.5, was tried. It was found unsuitable as a preparative procedure, however, since the products obtained were very crude and were found to be difficult to purify.

Further purification of the metabolites was followed by means of their characteristic ultraviolet absorption (Fig. 1). The optical density at the maxima (260 and 268 mμ) increased with purification, while end-absorption in the far ultraviolet region diminished rapidly. Both materials were puri-

fied by chromatography on a 120 × 20 mm. column of Florex XXS, prepared in ether, by using the gradient elution technique essentially as described by Moore and Stein (3). Tygon tubing is attacked by ether and was, therefore, replaced by polyethylene tubing. The mixing bottle (volume 175 ml.) contained ether, while the reservoir contained a mixture consisting at the start of 7 per cent ethanol and 93 per cent ether. The crude metabolite (50 to 70 mg.) was placed on top of the column in a minimal volume of ether. The flow rate was 30 ml. per hour. 5 ml. fractions were collected in test-tubes in a Technicon automatic fraction collector. Recoveries of 50 to 60 per cent of the material absorbing at 268 mμ were obtained.

A more convenient method that results in better recoveries has since been devised. The procedure utilizes a silicic acid-celite (2:1) column prepared in isooctane and a developing solvent consisting of 90 per cent isooctane and 10 per cent ethyl acetate. The size of the column, the flow rate, and the method of collecting the fractions are the same as in the method described above. The well defined yellow band of metabolite can be followed visually in this system. Recovery of material which absorbs at 268 mμ is essentially quantitative. Repetition of passage through either column does not appear to increase the purity of the product as judged by its ultraviolet absorption spectrum. The quantities of purified metabolites isolated per 24 hour urine specimen ranged from 3 to 6 mg. for the neutral and from 0.5 to 1 mg. for the acidic metabolite. The low recovery of the latter is due in part to destruction that occurred during its removal from the petroleum ether with sodium bicarbonate.

Characterization of Metabolites—Both metabolites are yellow oils which so far have resisted all attempts at crystallization. They are slightly soluble in water and in aliphatic hydrocarbons and readily soluble in all other organic solvents tested. Both substances have been shown to be considerably more polar than α-tocopherol or any of its known oxidation products. In the paper chromatographic system of J. A. Brown (4) with 20 per cent acetonitrile in water as the developing solvent, α-tocopherol and α-tocopherylquinone remain at the origin. The R_F of the neutral metabolite in this system is 0.60 and that of the acidic material is 0.85. These results suggested a reduction in size of the isoprenoid side chain. More conclusive evidence for this is represented by the molecular weight determinations (the Rast method with camphor as the solvent) and by the analyses.[3] For the neutral substance the empirical formula $C_{16}H_{20}O_4$ agrees best with the molecular weight of 267 (256 to 278)[4] and with the analytical results:

$C_{16}H_{20}O_4$. Calculated, C 69.55, H 7.25; found, C 69.34, H 7.29

[3] Analyses and molecular weight determinations were carried out by Dr. C. Tiedcke, Teaneck, New Jersey.

[4] The molecular weights represent averages of four determinations with a range of values between the limits shown in the parentheses.

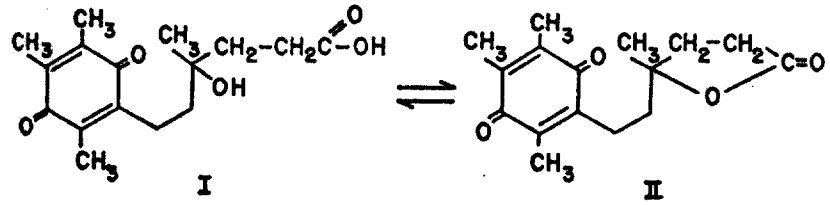
For the acidic material the molecular formula $C_{16}H_{22}O_5$ is most consistent with the molecular weight of 315 (307 to 326) and with the analytical data:

$C_{16}H_{22}O_5$. Calculated, C 65.31, H 7.48; found, C 65.22, H 7.18

These empirical formulae suggest that 13 carbon atoms have been removed from the molecule of α-tocopherol during its metabolism.

Both metabolites, after purification, exhibit the ultraviolet spectrum shown in Fig. 1. This spectrum is qualitatively identical with that of α-tocopherylquinone, and its molar extinction coefficient of 1.9 to 2.2 × 10^4, based on the calculated molecular weights, is in the range of the best values reported for that of α-tocopherylquinone (5). On the assumption that the same chromophoric groups are responsible for these two spectra, the metabolites may be considered to be reasonably pure. This is confirmed by the consistency of the analytical results and infra-red spectra of different samples obtained and purified by the methods described.

The structures postulated for the metabolites are 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone (I) and its γ-lactone (II).



I ⇌ II

Evidence for the various functional groups and the data on the preparation and properties of a crystalline derivative of the lactone will now be presented.

Evidence for Presence of Quinone Grouping—The ultraviolet absorption spectrum (Fig. 1) is characteristic of molecules containing the duroquinone (tetraalkyl *p*-benzoquinone) group, *e.g.* duroquinone (6), dihydrovitamin K [illegible], and α-tocopherylquinone (5). Further evidence for the presence of a quinone grouping was afforded by the infra-red spectra (Fig. 2, Curve A and Curve B), which for both metabolites showed a C=O stretching band at 1644 cm.$^{-1}$ (CCl_4), characteristic of α,β-unsaturated carbonyl groups (7). This band disappeared on reduction of the quinone.

Evidence for Presence of Carboxyl Group in Acidic Metabolite—The acidic material can be extracted from organic solvents with aqueous sodium bicarbonate which indicates a strongly acid group. Titration with 0.01 N sodium hydroxide in dilute aqueous ethanol with a Beckman pH meter gave a neutralization equivalent of 280 which is in good agreement with the calculated molecular weight and indicates the presence of one acid group per molecule. The pK of 4.9 is in the range of pK values usually observed for carboxyl groups. The carbonyl region of the infra-red spectrum of

this material (Fig. 2, Curve B) shows a band at 1710 cm.$^{-1}$ characteristic of dimeric carboxyl groups and a weaker band at 1755 cm.$^{-1}$ due to the C=O stretching of monomeric carboxyl groups. The hydroxy region shows the broad band from 3500 to below 3000 cm.$^{-1}$ (CS_2) typical of associated O—H stretching found in carboxylic acids (8, 9).

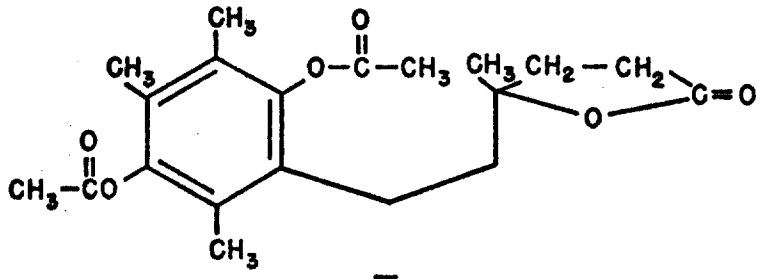
Evidence for Presence of Saturated γ-Lactone Ring in Neutral Metabolite—The neutral metabolite is converted readily into the acidic material by warming with dilute aqueous sodium hydroxide. This conversion is accompanied by considerable loss because of the instability of the metabolites in alkali. The acidic metabolite can be changed into the neutral one by standing for 24 hours with acetic anhydride. This ready interconversion suggested the presence of a lactone, anhydride, or ester grouping. This was confirmed by a positive hydroxamic acid test characteristic of compounds containing such "active acyl groups" (10). The test was quantitative in the best preparations, on the assumption that the color intensity of the hydroxamic acid-ferric ion complex is essentially independent of the types of groups attached to the hydroxamic acid group. The acidic metabolite did not give a positive test.

The infra-red spectrum of the neutral metabolite (Fig. 2, Curve A) was found to differ profoundly from that of the acidic material (Fig. 2, Curve B) in the "finger print" region (1400 to 650 cm.$^{-1}$) and the other regions as well. The broad band due to associated O—H stretching was absent. The carbonyl region again provided the most conclusive information. The 1611 cm.$^{-1}$ band ascribed to the α,β-unsaturated carbonyl groups of the quinone was still present, but the 1710 cm.$^{-1}$ and 1755 cm.$^{-1}$ bands due to the carboxyl group had been replaced by a band at 1780 cm.$^{-1}$. Such a band has been found to be typical of saturated γ-lactones (8, 9) and this band appears to rule out the presence of δ-lactones and esters, which generally absorb at 1750 to 1735 cm.$^{-1}$ (8, 9), anhydrides which have two bands at 1825 to 1810 cm.$^{-1}$ and at 1760 to 1748 cm.$^{-1}$ (9), α,β-unsaturated γ-lactones, which show two bands at 1786 to 1780 cm.$^{-1}$ and 1757 to 1755 cm.$^{-1}$ (8), and β,γ-unsaturated γ-lactones which absorb near 1800 cm.$^{-1}$ (9).

The absence of absorption at 220 mμ in the ultraviolet (Fig. 1) also makes unlikely the presence of an α,β-unsaturated lactone. Finally, unsaturation in the side chain is improbable because of the results of the analysis for hydrogen which would be decreased from 7.25 to 6.57 per cent by the removal of 2 hydrogen atoms. This difference is well outside of the limits of error for the analysis.

Crystalline Derivative of Neutral Metabolite—Reductive acetylation of the lactone with zinc, sodium acetate, and acetic anhydride by the procedure of John and coworkers (5) and recrystallization from

octane yielded a white crystalline compound, m.p. 95–98°. Its proposed structure is that of the lactone of 2-(3-hydroxy-3-methyl-5-carboxypentyl)



III

3,5,6-trimethylhydroquinone diacetate (III). Its molecular weight by the Rast method was 332 (319 to 343).

$C_{20}H_{26}O_6$.	Calculated.	C 66.29, H 7.18
	Found.	" 65.90, " 7.05

The ultraviolet absorption spectrum of this compound is identical with that of α-tocopherylhydroquinone diacetate (Fig. 3), as expected, since the

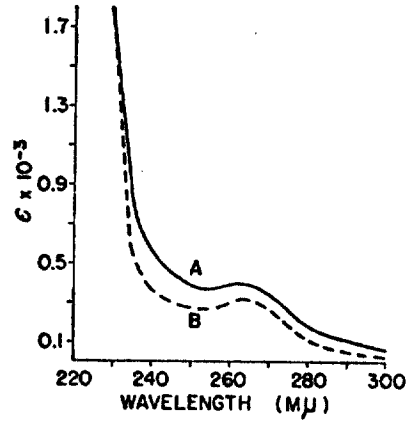


FIG. 3. Ultraviolet absorption spectra of the hydroquinone diacetate derivative of the lactone (Curve A) and of α-tocopherylhydroquinone diacetate (Curve B).

proposed structure would have the same chromophoric groups as the latter. The carbonyl region of the infra-red spectrum (Fig. 2, Curve C) shows that the α,β-unsaturated C=O band at 1644 cm.$^{-1}$ (due to the quinone group) has disappeared. The 1780 cm.$^{-1}$ band ascribed to the γ-lactone C=O is still present and a new band at 1764 cm.$^{-1}$ characteristic of enol acetates (8) has been introduced in agreement with the structure postulated for the derivative.

DISCUSSION

The two metabolites isolated from human urine appear to be identical with those found in the urines of rabbits given *d*-α-tocopheryl-5-methyl-C^{14}-succinate. The evidence indicates that in the rabbit these metabolites, predominantly in conjugated form, comprise approximately 90 per cent of the total urinary excretion products of α-tocopherol (20 to 30 per cent of the intravenous dose). No information is available yet for humans regarding either the fraction of absorbed α-tocopherol excreted in the urine or the portion of urinary products represented by these metabolites. If, in view of the large doses of α-tocopherol administered, we may assume a relatively low level of absorption from the gastrointestinal tract, the amounts of metabolites recovered from the urine represent a fraction of the absorbed vitamin E roughly comparable to the fraction of α-tocopheryl-5-methyl-C^{14}-succinate excreted in the urines of rabbits (2).

The relative amounts of lactone and hydroxy acid recovered may not reflect accurately the actual ratio of these metabolites excreted in view of the destruction of some of the hydroxy acid during sodium bicarbonate extraction[5] and the possibility of interconversion during the isolation procedure. Experiments in which the enzymatic hydrolysis was carried out directly on fresh urine indicate that the lactone is not merely an artifact of storage and ether extraction of urine at pH 1, since considerable quantities of lactone were demonstrated in the petroleum ether extract by paper chromatography (4) and by spectral analysis after sodium bicarbonate extraction. Moreover, when a sample of purified hydroxy acid was stored in aqueous solution at pH 1 overnight and then extracted continuously with ether, no lactone formation was observed.

The structures proposed for the metabolites suggest that the following changes have taken place in at least a portion of the absorbed α-tocopherol: opening of the chroman nucleus and oxidation to the quinone state, shortening of the isoprenoid side chain by 13 carbon atoms, oxidation of the terminal methyl group of the shortened side chain to a carboxyl group, and in the case of the neutral metabolite, lactonization of the carboxyl group with the tertiary hydroxy group formed as a result of the hydrolytic opening of the chroman nucleus. Another change, not evident in Structures I and II, is conjugation with glucuronic acid and perhaps to a lesser degree with other conjugating groups. The major portion of the urinary metabolites was excreted in conjugated form. The significance of these changes and the possible relationship they may bear to the alterations undergone in

[5] Both metabolites are unstable in alkali as a result of the presence of the quinone group. However, the lactone is not exposed to the destructive action of the alkali since it remains in the petroleum ether.

tocopherol in the formation of its metabolically active form are being investigated.

Work is in progress to determine the structures of the conjugated forms of these metabolites. On the basis of the results of Hoskin *et al.* concerning the nature of the urinary excretion products of menadione (11), it is suggested that the metabolites may be excreted as the corresponding hydroquinones with conjugating groups attached to one or both of the phenolic

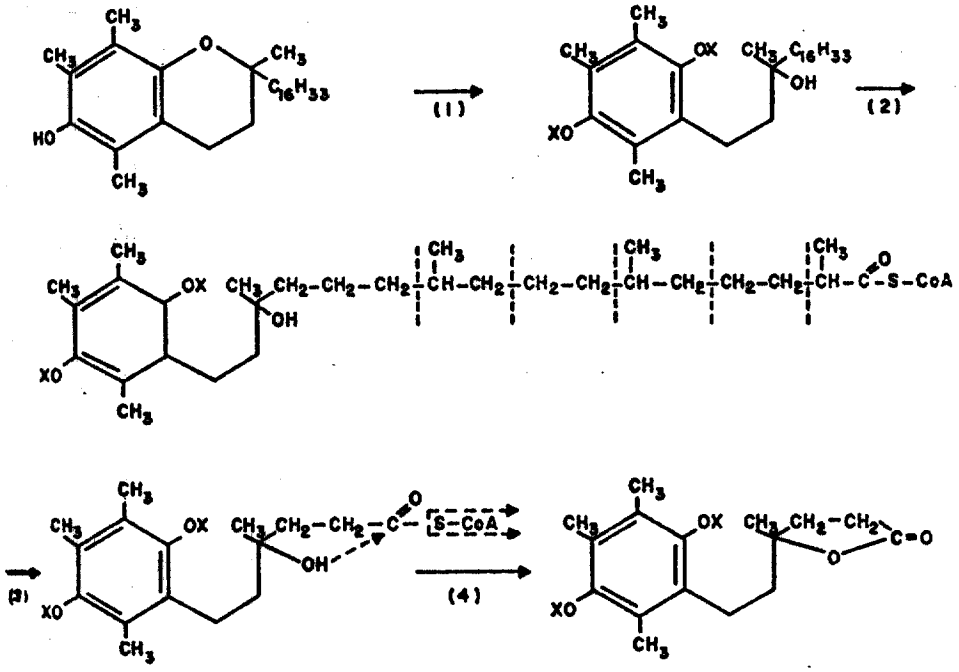


FIG. 4. Hypothetical scheme for the biological formation of the metabolites. X may be a hydrogen atom or a glucuronic acid residue.

hydroxy groups. The hydroquinones formed on hydrolysis would be very susceptible to oxidation by air, forming the observed quinones rapidly.

The same metabolites were obtained from urines of normal humans as from those of patients afflicted with muscular disorders, such as progressive muscular dystrophy, myotonia dystrophica, and amyotrophic lateral sclerosis. No conclusions regarding the relationship, or lack thereof, of vitamin E to muscular disorders can be drawn until quantitative data regarding the excretion of these metabolites become available and until more is known about the relation of these excretion products to the biologically active form of α-tocopherol.

A hypothetical scheme for the biological formation of the metabolites is shown in Fig. 4. In the scheme the chroman ring is opened hydrolytically, and the hydroquinone is conjugated (Step 1). The terminal methyl group of the side chain is oxidized to a carboxyl group, and the resulting fatty acid is conjugated with coenzyme A (Step 2) and degraded by β-oxidation (Step 3). The termination of β-oxidation 2 carbons short of completion may be explained by a nucleophilic attack by the hydroxy group on the acyl coenzyme A to form the stable γ-lactone, pushing out the coenzyme A molecule and thus terminating β-oxidation (Step 4). This last reaction is analogous to the biological acylation of hydroxy or amino groups by acyl coenzyme A derivatives (12, 13) and is, furthermore, favored by the formation of the stable 5-membered ring. Subsequent hydrolysis of some of the lactone may account for the acidic metabolite. Evidence for the oxidation of terminal methyl groups of aliphatic hydrocarbons to carboxyl groups has been obtained by Bernhard and coworkers (14, 15), who injected deuterium-labeled aliphatic hydrocarbons into rats and found the label in the fat. The degradation of the side chain has an analogy in the conversion of the side chain of cholesterol to that of cholic acid (16). If the correct mechanism in this case involves steps analogous to Steps 2 and 3 of Fig. 4, termination of β-oxidation 2 carbons short of completion is again observed. A different termination step must be postulated here, however, since the hydroxy group is missing.

SUMMARY

Two metabolites of α-tocopherol, largely in conjugated form, were found in the urines of humans ingesting large quantities of vitamin E. After hydrolytic removal of the conjugating groups, the metabolites were purified and characterized. Chemical, spectroscopic, and analytical evidence is presented for the formulation of their chemical structures as 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone and its γ-lactone. A crystalline derivative of the lactone was prepared. A hypothetical pathway for the biological formation of the metabolites is discussed.

Addendum—Since the proofs were received by us, we have obtained lactone in crystalline form. It crystallizes from 40 per cent ethanol as yellow glistening platelets.

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Effect of Certain Tocopherols and Other Antioxidants on the Utilization of β-Carotene for Vitamin A Storage [1]

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Introduction

Several observations suggest that excess tocopherol may interfere with the utilization of β-carotene by the rat for growth (1,2) or for the formation of vitamin A stores (3,4). Two mg. of α-tocopherol/day appears to be sufficient to depress the growth of rats fed limiting amounts of β-carotene, while 5–10 mg. of α-tocopherol or α-tocopheryl acetate/day limits the accumulation of vitamin A in rats given moderate amounts of β-carotene. This latter effect was not observed when the tocopherol and carotene were fed 8 hr. apart or when preformed vitamin A was substituted for β-carotene (3). The present report deals with compounds closely related to α-tocopherol: γ-tocopherol, δ-tocopherol, and α-tocopheryl acetate. The supplementary antioxidants, hydroquinone, ascorbic acid, and diamylhydroquinone, were also fed for possible effects on the activity of β-carotene.

Methods

Weanling rats of the Holtzman strain were placed on a diet low in vitamin A (3) until a deficiency state was recognized by eye signs and a failure to gain weight over a period of 7 days. The rats were then divided into groups of four to five comparable in weight, and were given daily either 40 μg. of β-carotene of 40 International Units (I.U.) of vitamin A as in previous experiments (3). Various amounts of the tocopherols were also administered with the vitamin A source. The daily doses of vitamin A, β-carotene, or tocopherol were each fed in 3 drops of cottonseed oil from calibrated droppers. In certain experiments either 0.1% of hydroquinone or 0.5% of ascorbic acid was added to the diet, or a supplement of 2.5 or 5 mg. of diamyl-

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hydroquinone was given alone or with α-tocopherol. After 15 days of supplementation the animals were killed by decapitation, and the livers and kidneys were analyzed for vitamin A with $SbCl_3$ as described previously (5,6).

In absorption studies, weanling rats were depleted of vitamin E for 2–3 months on a diet composed of glucose monohydrate (Cerelose) 73, casein 18, salts 4, lard 5, with vitamins added at the following levels in mg./kg. of diet: thiamine 3, riboflavin 2, pyridoxine 2.5, calcium pantothenate 7.5, and choline 1000. Each rat also received 2 drops of halibut liver oil every 4 weeks. Following approximately 18 hr. fasting, the rats were given 7.5 mg. of α-tocopherol or an equimolar amount of α-tocopheryl acetate in 3 drops of cottonseed oil and fed 2–3 g. of ration. After intervals of 0, 2, 5, 8, and 12 hr., the rats were killed and the tocopherol content of the small intestine was determined by a modification of the Emmerie-Engel method (7,8,9).

Results

Storage of Vitamin A in the Tissues

The average quantity of vitamin A retained in the livers and kidneys of rats fed β-carotene with only 0.5 mg. of α-tocopherol/day ranged from 33.8 to 59.4 μg. in the various series, and corresponding amounts were stored when the daily supplement was 2.5 mg. of α-tocopherol except in series 3 in which this level of tocopherol appeared to depress the storage of vitamin A somewhat (Table I). Supplementation with amounts of α-tocopherol in excess of 2.5 mg./day depressed the amounts of vitamin A in the livers and kidneys of rats fed carotene, thus confirming earlier observations (3).

When 12.5 mg. of γ-tocopherol/day was the supplement given with carotene, the depression in hepatic and renal vitamin A was also marked. This depression was equal to that due to α-tocopherol in one series (Table I, series 3) and was definite though inferior to the α-compound in a second (Table I, series 2). The accumulation of vitamin A was also diminished by 7.5 mg. of γ-tocopherol, and the degree of depression was similar to that due to α-tocopherol in two series (Table I, series 3 and 4) and inconclusive in a third (series 1) in which there was a depression at 2.5 mg./day but not at 7.5 mg./day. The over-all conclusion is that γ-tocopherol affects carotene metabolism similarly to α-tocopherol, but is probably somewhat less active. On the other hand, δ-tocopherol was inactive at 2.5 and 7.5 mg./day (Table I, series 1).

α-Tocopheryl acetate was found to lower the stores of vitamin A in carotene-fed rats whenever the dose of the ester exceeded 2.5 mg. equiv. of tocopherol/day, and in most experiments the esterified com-

pound was more active mole for mole than the free tocopherol: e.g., in series 4 (Table I) rats on 7.5 mg. of the acetate stored only 17.6 μg. of vitamin A as compared to 28.3 μg. by rats receiving free α-tocopherol. The presence of hydroquinone or ascorbic acid in the ration during supplementation with carotene and either 2.5 or 7.5 mg. of α-tocopherol/day was without effect on the activity of the tocopherol; the

TABLE I

Storage of Vitamin A in Rats Fed β-Carotene[a] with Various Tocopherols and Other Antioxidants

Supplement	Total vitamin A (liver and kidneys)			
	Series[b] 1	2	3	4
	μg.	μg.	μg.	μg.
None	52.0 ± 4.4[f]			
0.5 mg. α-tocopherol	59.4 ± 7.1	50.2 ± 5.1	33.8 ± 5.0	34.7 ± 4.2
2.5 mg. α-tocopherol	53.7 ± 3.8		37.7 ± 7.5	19.5 ± 6.1
2.5 mg. γ-tocopherol	46.2 ± 5.7			
2.5 mg. δ-tocopherol	52.2 ± 3.1			21.5 ± 1.8
2.5 mg. α-tocopheryl acetate			40.5 ± 2.2	
2.5 mg. α-tocopherol + 5 mg. DAHQ[c]			36.9 ± 4.0	
2.5 mg. α-tocopherol + 0.1% HQ[d] in diet			36.5 ± 2.1	
2.5 mg. α-tocopherol + 0.5% ascorb.[e] in diet			32.8 ± 7.0	28.3 ± 2.5
7.5 mg. α-tocopherol	38.1 ± 4.0	31.2 ± 2.7	32.2 ± 4.3	27.1 ± 2.4
7.5 mg. γ-tocopherol	54.9 ± 3.9			
7.5 mg. δ-tocopherol	52.1 ± 6.1			
7.5 mg. α-tocopheryl acetate		24.3 ± 3.8	15.9 ± 2.1	17.6 ± 5.1
7.5 mg. α-tocopherol + 2.5 mg. DAHQ				21.5 ± 3.9
7.5 mg. α-tocopherol + 5.0 mg. DAHQ			22.0 ± 2.6	
7.5 mg. α-tocopheryl acetate + 5.0 mg. DAHQ			22.1 ± 2.0	
2.5 mg. DAHQ				26.4 ± 3.6
5.0 mg. DAHQ			27.2 ± 0.9	
7.5 mg. α-tocopherol + 0.1% HQ in diet		32.5 ± 4.5		
7.5 mg. α-tocopherol + 0.5% ascorb. in diet		31.7 ± 2.6		
12.5 mg. α-tocopherol		26.3 ± 2.0	20.8 ± 2.4	
12.5 mg. γ-tocopherol		35.1 ± 0.4	22.5 ± 3.3	
15 mg. α-tocopherol	31.1 ± 5.5			

[a] Forty micrograms of β-carotene daily.

[b] Series 1 consisted of female rats; the other series, of males.

[c] DAHQ = diamylhydroquinone.

[d] HQ = hydroquinone.

[e] Ascorb. = ascorbic acid.

[f] Standard error.

rats in both groups stored amounts of vitamin A almost identical with those receiving only α-tocopherol (Table I, series 2 and 3). Hydroquinone or ascorbic acid apparently do not spare tocopherol or other substances in the lipides of the digestive tract (10).

When the fat-soluble antioxidant, diamylhydroquinone (11), was fed at 15 mg./day, the rats developed diarrhea in 2–3 days; at 5 mg./day

TABLE II

Storage of Vitamin A in Rats Fed Vitamin A[a] with Various Tocopherols and Other Antioxidants

Daily supplement	Total vitamin A (livers and kidneys) Series[b] 1 μg.	Series 3 μg.
None	29.6	
0.5 mg. α-tocopherol		21.2
12.5 mg. α-tocopherol		22.0
15.0 mg. α-tocopherol	35.6	
12.5 mg. γ-tocopherol		23.7
7.5 mg. α-tocopheryl acetate		27.8
5 mg. DAHQ[c]		29.7

[a] Forty International Units of vitamin A daily as diluted halibut liver oil.
[b] Series 1 consisted of female rats, series 3 of males.
[c] DAHQ = diamylhydroquinone.

the rats still did not readily accept the compound, but the toxic manifestations largely disappeared. Diamylhydroquinone did not depress the utilization of carotene when fed with 2.5 mg. of α-tocopherol (Table I, series 3), but when 7.5 mg. of α-tocopherol was fed, the further addition of diamylhydroquinone resulted in smaller stores of vitamin A than in the group receiving only tocopherol (Table I, series 3 and 4). Diamylhydroquinone also appeared to minimize the storage of vitamin

TABLE III

Tocopherol in the Small Intestine of the Rat After Supplementation With α-Tocopherol or α-Tocopheryl Acetate

(μg./small intestine)

Supplement	Time after supplementation 0 hr. μg.	2 hr. μg.	5 hr. μg.	8 hr. μg.	12 hr. μg.
7.5 mg. of α-tocopherol	184	346	383	248	552
	76	342	296	223	269
	66	228	392	423	596
			1010	557	
Average	109	305	516	363	472
8.3 mg. of α-tocopheryl acetate		186	625	605	468
		65	277	717	486
		817	115	184	366
			318	824	
Average		356	334	582	440

A by itself, although all of these experiments were probably complicated by the poor acceptability of the compound.

When the source of the vitamin A was fish liver oil, storage was unaffected by high amounts of α-tocopherol, γ-tocopherol, α-tocopheryl acetate, or of diamylhydroquinone, and in some experiments storage appeared to be enhanced slightly by these antioxidants (Table II).

Vitamin E in the Intestinal Wall

In a preliminary trial in which only α-tocopherol was fed, the largest amounts of tocopherol were recovered from the intestine between 2 and 5 hr. after supplementation, although appreciable quantities were still present after 12 hr. Similar results were obtained when both the free alcohol and tocopheryl acetate were fed (Table III), although variations within groups were large. Five hours after supplementation with α-tocopherol the amount of tocopherol in the intestinal wall reached a maximum of 516 μg.; when the ester was fed, the maximum amount, 582 μg. of tocopherol, was reached after 8 hr. Twelve hours after supplementation there was still more than 400 μg. of tocopherol in the intestinal wall of both groups.

Discussion

Although a number of observations point to the desirability of vitamin E in doses exceeding those necessary to prevent frank signs of deficiency (12,13,14), the present results again emphasize that there is such a thing as an optimal dose of vitamin E, and that the range of optimal activity is not particularly wide (2,3). For the accumulation of vitamin A in the tissues of rats fed moderate amounts of carotene, the optimal amount of tocopherol appears to range from 240 μg./day, the amount consumed on a diet containing 5% of cottonseed oil, to somewhat less than 5.5 mg./day (3). This interference with the utilization of carotene might be regarded as one aspect of a hypervitaminosis E. α-Tocopheryl acetate appeared to be about 67% more active than the free alcohol in suppressing the accumulation of vitamin A from carotene, and the relative activities of ester to alcohol are also of this order of magnitude when the biological endpoint is the mean fertility dose (11). On the other hand, γ-tocopherol was almost as active as α-tocopherol in minimizing the utilization of carotene (Table I) although its activity in the fertility test is only about 1/12 that of

α-tocopherol (15). The effect of γ-tocopherol on carotene, therefore, appears to be more like its antioxidant potency *in vitro* than its potency as an antisterility vitamin.

High and Day (4) have pointed out that the effect of tocopherol on carotene parallels its effect on fats; small amounts inhibit autoxidation while large amounts enhance it (16,17). The concentration of free tocopherol necessary for enhanced autoxidation is, however, almost certainly greater than that in the intestine when tocopheryl acetate is fed, for tocopherol is an antioxidant only in the free form (18).

The intestinal wall would therefore appear the more likely locus of the effect. The amounts of tocopherol found in the intestinal wall after the ingestion of free tocopherol or tocopheryl acetate were quite similar, but the time of maximum concentration was later for the ester than for the free alcohol. It is possible that the time of maximum absorption of carotene (19) coincides more closely with that of tocopheryl acetate than of free tocopherol, and that the relatively high activity of the ester in suppressing vitamin A storage depends on this circumstance. The mechanism of suppression could be either a diminished absorption of carotene, or an interference in the reaction by which carotene is converted into vitamin A (3).

Acknowledgment

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Summary

1. As did α-tocopherol, high doses of γ-tocopherol or α-tocopheryl acetate diminished the storage of vitamin A in the livers and kidneys of rats fed moderate amounts of β-carotene. γ-Tocopherol was nearly equal to α-tocopherol while α-tocopheryl acetate was somewhat more active than the free vitamin. Hydroquinone, ascorbic acid, and diamylhydroquinone failed to increase the activity of α-tocopherol, but diamylhydroquinone itself slightly depressed the storage of vitamin A.

2. When preformed vitamin A was fed, tissue stores of the vitamin were not affected adversely by any of the antioxidants.

3. The maximum concentration of tocopherol in the intestinal wall was observed 5 hr. after the ingestion of free α-tocopherol and 8 hr. after the ingestion of α-tocopheryl acetate. The amounts found at these times were, however, quite similar.

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THE EFFECT OF VITAMIN E ON THE OVARIAN FUNCTION

Report XI

Incretion and Avitaminosis

Article by: Szarka, A. - (Debrecen University, Debrecen, Hungary)

Vitamin E was identified by Evans & Burr /1/ as a component of the wheat embryo, which, when added to otherwise incomplete food, supplements it for rats. Deficiency in the vitamin causes sterility in rats manifested by the fact that females fed for an extensive period of time without it, conceive but do not carry their pregnancy to completion. The fetus dies in the second half of the pregnancy, it is macerated and reabsorbed. The sterility of the breed develops in most cases over a longer period of time, frequently in the second generation. Males also develop sterility in case of vitamin E deficiency after a certain time; it appears as a form of azoospermia. The phenomena can be reversed by wheat embryo or its ethereal extract. The substance has been prepared from other grains and green leaves also. The authors showed that the unsaponifiable fraction freed from cholesterin and sitosterin contains the effective ingredient. The latter is highly resistant to heat, light and chemicals. With the exception of the propagation of animals, vitamin E deficiency has no effect on the state of health or the growth of animals. It is therefore a substance connected only with genital functions.

The author's institute, under the direction of Prof. F. Vezar, studied the problem of the relationship of vitamin effects and incretion /2/. Relationships were demonstrated in several instances and it appeared logical to search for a connection between vitamin E and genital hormones also. For this, two paths are available: (1) to investigate whether vitamin E is capable of producing effects proper to genital hormones,

specifically the ovarian hormone, and (2) whether it is possible to compensate dietary vitamin E deficiencies with ovarian hormones. The author chose the first approach and therefore, in the following, the possibility of obtaining effects with vitamin E exercized otherwise only by ovarian hormones shall be investigated. For the control of the hormone effect, the biological reactions of rats described by Allen and Doysi /3/ and Frank /4/, were used, Long & Evans /5/ and Papanicolau & Stockard /6/ found that with rodents, a parallel characteristic cyclic change occurs with each ovarian cycle in sexually mature animals. Characteristic histological changes take place in the vaginal membrane, easily demonstrated on smears of the content of the vagina. These cyclic variations of genital secretions are missing if the ovary is not yet or no longer functioning. Allen & Doisy showed that the phenomenon can be produced in infantile, senile or castrated animals by injecting ovarian hormones. A simple microscopic study of the vaginal smear of rats or mice thus suffices to determine the content in ovarian hormones or similarly effective substances of a preparate.

The author therefore tested extracts containing vitamin E for their ability to induce estrus in infantile rats.

Methodology:

The initial material was wheat embryo. It was extracted with ether. The oil was saponified with alcoholic potassium hydroxide. The unsaponifiable fraction contains vitamin E. Sitosterin (an isomer of cholesterin) is then removed with digitonin or pentane. In the present experiments, all 3 fractions were utilized: 1. etheral extract (oil), 2. unsaponifiable portion of the foregoing, 3. sytosterin fraction of the foregoing.

The ovarian cycle is examined on smear preparates. The following stages are distinguishable: if the vaginal secretion is transparent, mucous and only a few leukocytes and epithelia are present in the field of

view, no estrus is present. In pre-estrus or estrus the vaginal membranc swells, the upper-most layer of epithelial cells is expelled. Numerous nucleus-containing epithelial cells appear in the vaginal secretion and leukocytes characteristic of the rest stage (dioestrum) disappear. In fully developed estrus, numerous horny epithelial cells are found; they adhere to each other in groups. According to Laqueur /7/, the reaction is positive if the leukocytes disappear. This occurs when the vaginal membrane has thickened both in the pre-estrus and the estrus. Simultaneously, nucleus-containing epithelial cells appear in large numbers. They may not yet be horny, or only partially so. If no leukocytes, not more than 20% nucleus-containing epithelial cells and at least 80% horny epithelial cells are present, the result is designated ++. If a maximum of 4% leukocytes and more than 20% nucleus-containing epithelial cells are present and the number of horny epithelial cells is correspondingly reduced, the result is marked +. The secretion is negative if it consists only of mucus with few cellular elements, mainly leukocytes and max. 1-2% epithelial cells. It is marked -. A number of transitions exists between the + and - cases; they are uncertain and are designated by $\pm$. In such cases, there are at least 50% nucleus-containing epithelial cells, in addition to leukocytes. Such observations indicate a certain reaction but cannot be used as proof.

Another effect of the ovarian hormone is the opening of the vagina. The suthor observed the effect regularly but considers it significant only when accompanied by characteristic changes in the secretion.

The experimental animals consisted of 3-5 weeks old (infantile) white rats. In addition to these, control animals were used in all of the experiments. The latter were either brethren of the experimental animals, or, if not possible, animals of the same size and age, originating in the same breed. All control animals received the same food and were

maintained under identical conditions.

All three preparations were administered both per os and subcutaneously. A total of 42 rats were used; 13 were control animals.

Experiments:

The experiments are summarized in Table 1. They are discussed in groups, according to the differential treatment. Each experimental series included control animals of the same age, which received the same treatment. The results are shown in Table 2.

Table 1

Tabelle 1.

Versuchsreihe	Nummer	Gewicht in g	Erhielt Subst.	Erhielt Wohin	Menge täglich in g	Von — bis	Reaktion am Tage der Behandlung 2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Endgewicht in g	Resultat
1a	7	23,5	acth. ext.	per os	1	18. I.—30. I.	·	·	·	·	·	·	·	+	+	—	—	·	·	·	·	·	·	positiv
1a	8	23	acth. ext.	per os	1	18. I.—30. I.	·	·	·	·	·	·	·	±	±	—	—	·	·	·	·	·	·	unsicher
1b	3	23	acth. ext.	s. c.	2×0,5	21. I.—28. I.	·	·	·	·	·	·	+	—	·	·	·	·	·	·	·	·	·	positiv
1b	4	22	acth. ext.	s. c.	2×0,5	21. I.—28. I.	·	·	·	·	·	·	±	—	·	·	·	·	·	·	·	·	·	unsicher
2a	14	20,5	unversfb.	per os	0,25	31. I.—14. II.	·	·	·	·	·	+ +	·	·	·	±	·	·	·	±	·	·	·	stark positiv
2a	15	19,5	unversfb.	per os	0,25	31. I.—14. II.	·	·	·	·	·	+ +	·	·	·	±	·	·	·	±	·	·	·	stark positiv
2a	17	26,0	unversfb.	per os	0,25	12. II.—19 II.	·	·	·	—	+	+	·	·	—	·	·	·	—	·	·	·	·	positiv
2a	18	21	unversfb.	per os	0,25	12. II.—19. II.	·	·	·	+ +	+ +	—	·	·	—	·	·	·	—	·	·	·	·	stark positiv
2a	19	25,5	unversfb.	per os	0,25	12. II.—19. II.	·	·	·	—	—	—	·	·	—	·	·	·	—	·	·	·	·	negativ
2a	29	21	unversfb.	per os	0,30	25. IV.—4. V.	·	·	·	·	·	±	+	+	+ +	·	·	·	·	·	·	·	·	stark positiv
2a	30	26	unversfb.	per os	0,30	25. IV.—4. V.	·	·	·	·	·	±	+	+	+ +	·	·	·	·	·	·	·	·	stark positiv
2a	31	27	unversfb.	per os	0,30	25. IV.—4. V.	·	·	·	·	·	—	±	+	+	·	·	·	·	·	·	·	·	positiv
2a	32	22	unversfb.	per os	0,30	25. IV.—9. V.	·	·	·	·	·	·	±	±	±	±	—	±	·	±	—	—	·	unsicher
2a	33	19,5	unversfb.	per os	0,30	25. IV.—9. V.	·	·	·	·	·	·	±	±	—	—	·	—	—	—	—	—	·	unsicher
2a	34	20,0	unversfb.	per os	0,30	25. IV.—9. V.	·	·	·	·	·	±	—	·	—	—	·	—	·	—	—	—	·	unsicher
2a	35	18	unversfb.	per os	0,30	25. IV.—9. V.	·	·	·	·	·	·	·	—	—	—	—	—	—	—	—	—	·	negativ
2a	39	28	unversfb.	per os	0,30	23. IV.—9. V.	·	·	·	±	±	·	+	—	·	·	·	—	±	±	±	—	·	positiv
2a	40	30	unversfb.	per os	0,30	23. IV.—9. V.	·	·	·	±	±	·	+	—	·	·	·	—	±	+	—	—	·	positiv
2b	9	27	unversfb.	s. c.	0,5	21. I.—28. I.	·	·	·	·	+	+ +	+	—	·	·	·	·	·	·	—	—	·	stark positiv
2b	10	26,5	unversfb.	s. c.	0,5	21. I.—28. I.	·	·	·	·	+	+	+	—	·	·	·	·	·	·	—	—	·	positiv
2b	11	24	unversfb.	s. c.	0,5	21. I.—28. I.	·	·	·	·	—	—	—	—	·	·	·	·	·	·	—	—	·	negativ
3a	26	27	sitost.frei	per os	0,5	1. I.—7. I.	·	·	·	·	+	+ +	±	·	·	—	·	·	·	·	·	—	·	stark positiv
3a	27	31	sitost.frei	per os	0,5	1. I.—7. I.	·	·	·	·	—	±	±	·	·	—	·	·	·	·	·	—	·	unsicher
3b	22	24,5	sitost.frei	s. c.	0,30	1. I.—7. I.	·	·	·	·	·	·	·	—	·	—	·	±	·	·	·	·	·	unsicher
3b	23	25,5	sitost.frei	s. c.	0,30	1. I.—7. I.	·	·	·	·	·	·	·	±	+ +	+	·	±	·	·	·	·	·	stark positiv
3c	42	19,0	sitost.arm	s. c. u. p. os	0,25	3. V.—5. V.	·	±	+	+ +	+ +	+	±	—	·	·	·	·	·	·	·	·	·	stark positiv
3c	43	20,5	sitost.arm	s. c. u. p. os	0,25	3. V.—5. V.	·	±	+	+	+	+	±	—	·	·	·	·	·	·	·	·	·	stark positiv
3c	44	21	sitost.arm	s. c. u. p. os	0,25	11. V.—14. V.	·	·	±	±	+	+	±	±	·	·	·	·	·	·	·	·	·	positiv
3c	45	19,5	sitost.arm	s. c. u. p. os	0,25	11. V.—14. V.	·	·	±	±	±	+	+	±	·	·	·	·	·	·	·	·	·	positiv

Key: 1. Number of series; 1. Number; 3. Weight in g; 4. Received; 5. Substance; 6. How; 7. Daily amount in g; 8. From-to; 9. Reaction on the day of treatment; 10. Final weight in g; 11. Result; 12. Uncertain; 13. Strongly positive.

Table 2 - Controls

Tabelle 2. *Kontrollen.*

Nr.	Gew. in g am Anfang (2)	Gew. in g am Ende (3)	Gehört zu Nr. (4)	Resultat (5)	Nr.	Gew. in g am Anfang	Gew. in g am Ende	Gehört zu Nr.	Resultat
1	20,0	28,9	3, 4	0	20	27,5	35,2	17, 18, 19	0
2	19,8	29,6	3, 4	0	21	26,5	29,2	17, 18, 19	0
12	23,0	32,0	9, 10, 11	0	28	30,0	37,0	26, 27	0
13	20,0	35,0	9, 10, 11	0	41	21,0	26,0	28—35	0
25	28,5	29,8	22, 23	0	42	19.0	27,0	28—35	0
5	25,0	—	7, 8	0	46	21,5	24,0	42, 43	0
6	27,0	—	7, 8	0	47	22,0	25,5	44, 45	0
16	21,3	33,0	14, 15	0					0

Key: 1. Weight in g. at; 2. start; 3. eng; 4. belongs to; 5. result.

1a. Ethereal Extract (oil) given per os:

Animals 7 and 8 were given the substance in amounts of 1 g/day, mixed in their food. No. 7 showed positive reaction, No. 8 a negative reaction. The animals otherwise exhibited normal development.

1b. Same Substance, Subcutaneous:

No. 3 and No. 4 were given twice daily 0.5 ccm for 8 days. The animals reacted poorly to the administration of oil. They lost weight and perished in 8-10 days. Prior to exitus, however, No. 3 showed + reaction and No. 4 a reaction of $\pm$ and the vagina opened. The controls survived, their vagina remained closed.

2a. Unsaponifiable Portion, Per Os:

This proved to be substantially more effective. With per os administration, positive reactions were obtained in approx. 75% of the cases without appreciable effect on the state of health and growth of the

animals. However, not all preparations were equally effective. It was necessary to prepare the oil from different shipments of wheat embryo and the lots were not uniformly pure and valuable. Positive reactions occurred on the 3rd to 8th day and lasted 1-2 days, simarily to the normal estrus. Termination of the feeding or injections lead to a cessation of the estrus.

The following 15 animals received the substance per os: No. 14, 15, 17, 18, 19 0.25 g/day, No. 29, 30, 31, 32, 34, 39, 40 0.30 g/day. Of these animals, five were ++, four +, 4 ±; 2 -.

Two died 10 days after the start of the experiment. Five animals served as controls. All remained negative.

2b. Same Substance, Subcutaneous:

No. 9, 10, 11 received the fraction subcutaneously, once daily 0.5 ccm; No. 9 was ++, No. 10 +, No. 11 remained negative. The two controls, No. 11 and 12, were negative.

3a. Unsaponifiable Portion Free of Sitosterin, Per Os:

The substance was given in doses of 0.5 g in milk to No. 26 and 27. The first was ++, the latter ±. The control animal (28) remained negative.

3b. Administered Subcutaneously:

Two animals (22, 23) received once daily 0.3 ccm. The first gave an uncertain reaction, the latter ++. The control remained negative (25).

3c. To 4 animals (42, 44, 45) a preparation was given which was merely low in sitosterin, but not completely free of it (removed only with pentane). The animals received the substance for 3 days in doese of 0.25 g per os and on one day 0.15 g twice, subcutaneously. All developed estrus, 1 with ++, 3 with + reaction. Control animals were 46 (with 42, 44) and 47 (44, 45). The animals remained absolutely negative.

Discussion:

Positive reactions were induced with different preparations containing vitamin E in a total of 29 infantile rats. Nine were strongly positive (++), 10 positive (+), 7 were uncertain (±) and only 3 definitely negative (-).

The 3 negative experiments were in part the result of premature death, i. e. the toxic effect of the oil, so that their significance is slight. It may therefore be considered proven that an excess of vitamin E induces estrus in infantile rats. Vitmain thus has the same effect on infantile animals as ovarian hormone.

The pheomenon may be explained in a number of ways. (1) It is conceivable that vitamin E is a structural component of ovarian hormone. If the overy is missing, the substance is not produced so that the effect should not take place in castrated animals, regardless of the amount of vitamin E supplied. (2) It is also conceivable that the vitamin should be considered a stimulant rather than a component of the ovary. Its administration increases the incretion of the ovary in the same manner as hypophysis hormones. As is known, premature maturity can be obtained in infantile animals with the latter, while this is not true for castrated animals (Aschheim & Zondek, /8/). Hypophysis hormones thus also act by way of the ovary, by stimulating it to cyclic activity. (3) It should be recalled that according to Evans, the anterior lobe of the hypophysis is one of the organs of the animal body richest in vitamin E, so that it is possible that the effect of the latter occurs by way of the hypophysis. Investigation in this respect are under way.

Summary:

Estrus was induced in infantile rats by the feeding of substances high in vitamin E or by their subcutaneous injection. The vitamin was prepared from wheat embryo and used, in part, in the form of an etheral extract, as an unsaponifiable fraction or as an unsaponifiable fraction free of sitosterin. Of 29 infantile animals, estrus appeared in 19. All of the 13 control animals remained negative.

THE EFFECTS OF HYPO- AND HYPER-VITAMINOSIS E ON LUNG TUMOR GROWTH IN MICE*

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Introduction

Over a decade ago, several workers investigated the effects of vitamin E on tumor growth in mice and rats. Published reports of this work revealed considerable discrepancy in their results. Some workers reported that hypervitaminosis E in the mouse retarded tumor growth,[1,2,3,4,5,6] while other investigators found that tumor growth was unaffected by vitamin E.[7,8,9,10,11,12]

The rationale of this work was based largely on the observation of other earlier workers that vitamin E in some obscure manner played a rôle in rapid cell proliferation.[13,14,15,16] Because of the lack of uniformity of results among previous workers, it was decided to reinvestigate this problem, using synthetic alpha-tocopherol instead of the vitamin E concentrates used by the earlier workers.

Procedure

Two hundred young strain "A" (Bar Harbor hereditary lung tumor strain) mice were used in the experiment. They were divided equally into four groups. Group 1 was maintained on a vitamin E-deficient diet. Group 2 received the deficient diet plus 2 mg. of synthetic alpha-tocopherol on alternate days. Group 3 was held on a normal stock diet. Group 4 had normal diet plus 2 mg. of alpha-tocopherol every other day. All animals received, subcutaneously, 1 mg. (1, 2, 5, 6) dibenzanthracene in $\frac{1}{4}$ cc. olive oil.

A severe diarrhea epidemic during the first month of the experiment depleted the colony to about half its original size. At the end of seven months, all of the remaining animals were autopsied. The number, size, distribution, and histologic type of the tumors were noted (TABLE 1).

Results

The incidence of lung tumors in the combined normal groups (Groups 2, 3, and 4) was 94.8 per cent. The deficient group showed an incidence of only 70.9 per cent.

Actual counts of the number of lung tumors showed that Group 2 (supplemented) had the largest number per animal, averaging 125.9. Group 4 averaged 81.0 tumors per animal; Group 3 had 71.7 per animal; and the deficient group (Group 1) had an average number of 45.6 tumors per animal.

There was a sex difference in the number of tumors of the lung. Male animals of all groups had nearly double the number of tumors per animal as the females of the corresponding group. Incidence of tumors was about the same in both sexes.

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TABLE 1

PERCENTAGE OF TUMORS IN "A" STRAIN MICE

Group	*Diet*	*Number animals autopsied*	*Lung tumors*	*Cysts*	*Subc.-tumors*
			%	%	%
1	E-deficient	24	70.9	75.0	33.3
2	E-deficient plus α-tocopherol	37	94.6	24.3	35.1
3	Normal	22	90.9	22.7	36.4
4	Normal plus α-tocopherol	19	100.0	36.8	47.4

On the dorsum of the shoulders at the site of the injection of the carcinogen, subcutaneous tumors appeared in some animals of all groups. They were somewhat more abundant, however, in the normal supplemented group (Group IV). In this group, 47.4 per cent of the animals had these subcutaneous tumors. In the remaining three groups, about the same incidence was noted; namely, 33.3, 35.1, and 36.4 per cent.

During the early course of the experiment, many of the animals of all groups developed a small cyst (usually about 1 cm. in diameter) at the site of the injection. The oily semi-fluid content of these cysts was suggestive of the injection media and, therefore, the question was raised whether or not the carcinogenic agent had been fully absorbed. These cysts frequently broke down and caused an ulceration of the skin which often involved a considerable area over the shoulders and forelegs. The cysts and skin lesions were more frequent in the deficient group. Therefore, the lower incidence and smaller number of tumors in the deficient group might possibly be due to poor absorption of the carcinogen and not due to the nutritional deficiency per se.

Summary

In a seven-month experiment on induced lung tumors in strain "A" mice, the following results were noted:

(1) The incidence of lung tumor was greatest in the hypervitaminosis groups.

(2) The average number of lung tumors per animal was significantly greater in the hyper-vitaminosis E groups. The deficient animals had the least number of lung tumors per animal.

(3) Male animals of all experimental groups invariably showed about twice the number of lung tumors per animal as the corresponding female of the same dietary group. No sex difference was noted in the incidence of the tumors.

(4) Subcutaneous tumors in the interscapular region at the site of injection of the carcinogen were more prevalent in the groups receiving excessive alpha-tocopherol supplementation and least prevalent in the E-deficient groups.

(5) The more frequent appearance of oil-filled cysts at the site of injection

in the deficient group suggests possibly poor absorption of the carcinogen, which may account to some extent for the low incidence of tumors in this deficient group.

(6) These findings suggest that vitamin E deficiency may reduce the incidence and number of lung tumors in Strain "A" mice as compared to the normal and hypervitaminosis E control animals.

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Fetal Resorption in the Rat as Influenced by Certain Antioxidants[1]

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Some prenatal mortality is perhaps a normal phenomenon accompanying gestation in all mammals. Yet there is a paucity of definitive data on total embryonic or fetal wastage as compared with the wealth of information on the pathologic, genetic, physiologic and nutritional causes of prenatal death. Of the literature which deals with this problem in man or domesticated animals, most of it deals with fetal mortality, either as stillbirths or abortions.

In the rat there are many factors that may increase fetal death and subsequent resorption as, insufficient protein (Barry, '20), elevated environmental temperature (Sundstroem, '27; Hsu, '48; MacFarlane, Pennycuik and Thrift, '57), vitamin deficiencies, e.g., biotin (Kennedy and Palmer, '45), pantothenic acid (Nelson and Evans, '46), pyridoxine (Nelson and Evans, '48), pteroylglutamic acid (Nelson, Asling and Evans, '52), tocopherol (Cheng and Thomas, '53) and riboflavin (Nelson et al., '56). Other causes of resorptions are ovariectomy (Haterius, '36), trypan blue injections (Wilson, '54), Lathyrus odoratus feeding (Walker and Wirtschafter, '56) and others. There is also evidence that loss due to resorption is not confined to any particular period of pregnancy but occurs throughout gestation (wild rat, Perry, '46; wild rabbit, Brambell, '48; mouse, Hollander and Strong, '50).

Because the rat resorbs rather than aborts an imperfect conceptus, a record of all implantations is retained in the uterine mucosa at the implantation sites. This record is represented by remnants of implantation ranging from resorbed fetuses to inconspicuous cicatricial plaques. A careful macroscopic examination of the incised term uterus will therefore supply data on total implantations as well as normal young.

Resorption data secured from the examination of nearly 500 pregnant rats involving approximately 5,000 fetuses and over 500 resorptions is herewith presented. In addition, we investigated the influence of certain maternally administered compounds, mostly antioxidants, on the incidence of fetal loss as compared to normal animals held on normal diets. These data are the outgrowth of a larger problem concerned with the possible value of these antioxidants and related compounds in the prevention or amelioration of lesions arising from neonatal anoxia.

MATERIALS AND METHODS

Walter Reed-Carworth Farms strain rats in their first gestation were used exclusively and weighed approximately 200 gm at time of breeding. After positive mating the females were randomly distributed into experimental and control groups and the former were given *per os* or directly into diet one of the various compounds listed in table 1. Twenty-two days after positive mating the pregnant rat was killed in 100% nitrogen and after 21 minutes the young delivered by caesarian section. Both horns of the uterus were completely incised and a careful macroscopic survey made for resorption sites. The resorptions were charted as to location in the respective region of the horns and classified as to degree of resorption. Seasonal variation in the number of resorptions were recorded.

RESULTS

In 126 normal untreated gravid animals 40.8% had one or more resorptions and 10.6% of all recognizable implantations had terminated in resorptions. Of the 29 dietary regimens used, animals receiving

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TABLE 1

Effect of antioxidants and other compounds on fetal resorption[1]

	Total dose	Per os or in diet	Litters	Implantations	Normal fetuses	Litters with resorptions	Resorptions
	gm					%	%
Butylated hydroxytolulene (BHT)	0.5	D	11	124	121	18.2	2.4
d-l,alpha tocopherol acetate	0.2	O	12	169	162	41.7	4.1
2,5-di-tert-amylhydroquinone	0.25	D	10	134	128	60.0	4.5
d gamma tocopherol	0.2	O	11	127	121	45.5	4.7
Butylated hydroxyanisole (BHA)	0.5	D	12	145	137	51.7	5.5
d gamma tocopherol	0.07	O	12	157	148	50.0	5.7
2,6-di-tert-butyl-4-methyl-phenol (DBMP)	0.5	D	11	140	131	54.5	6.4
Thiodipropionic acid	0.5	D	10	128	119	60.0	7.0
1,2-di-hydro-2,2,4 trimethyl-6-ethoxyquinoline (Santoquin)	0.25	D	12	147	136	33.3	7.5
1,2-di-hydro-2,2,4 trimethyl-6-phenylquinoline (Santoflex B)	0.25	D	11	139	128	54.5	7.9
Nordihydroguaiaretic acid (NDGA)	0.5	D	8	86	79	75.0	8.1
Pangamic acid sodium (vit. B_{15})	0.25	D	11	138	126	27.2	8.7
Thiamine hydrochloride	2.0	O	13	160	145	69.2	9.4
Ascorbic acid	2.0	O	14	146	132	71.4	9.6
Glucose	30% of diet	D	25	280	251	68.0	10.3
Normal			126	1430	1278	40.8	10.6
Thiamine hydrochloride	0.5	D	8	75	67	62.5	10.7
2,2-methylene bis(4-methyl-6-tertiary butyl phenol)	0.25	D	8	101	90	37.5	10.9
Polymerized 1,2-dihydro-2,2,4-trimethyl quinoline (Flectol H)	0.25	D	17	148	136	52.9	12.1
Cytochrome C	0.05	D	8	84	73	50.0	13.1
Glutathione	0.25	D	6	65	56	66.6	13.9
d-l,alpha tocopherol acetate	0.1	O	14	150	129	71.4	14.0
Ascorbic acid	0.5	D	20	235	199	70.0	15.3
N,N'-diphenyl-p-phenylene-diamine (DPPD)	0.5	D	23	275	233	60.8	15.3
Phenothiazine	0.25	D	10	89	75	70.0	15.7
2,2-methylene bis(4-ethyl-6-tertiary butyl phenol)	0.25	D	9	111	92	66.6	17.1
n propyl gallate	0.5	D	9	93	76	77.7	18.3
Paraminobenzoic acid (PABA)	0.5	D	9	105	80	77.7	23.8
Hydroquinone	0.5	D	10	105	77	100.0	26.8
Methylene blue	0.5	D	10	100	62	90.0	38.0

[1] For samples of antioxidants we are indebted to the American Cyanamid Company for 2,2-methylene bis (4-methyl-6-tertiary butyl phenol) and 2,2-methylene bis(4-ethyl-6-tertiary butyl phenol), Distillation Products Industries for dl-alpha tocopherol acetate and gamma tocopherol, the B. F. Goodrich Company for DPPD, the Monsanto Chemical Company for Santoflex B, Santoquin and Flectol H and Hale Laboratories for vitamin B_{15}.

one of 15 showed less resorptions (range 2.4–10.1%) than the controls, while rats receiving one of the other 14 diets showed an increased percentage of resorptions (10.7–38.0%) (table 1). In the dosage given none of these compounds appeared to be toxic to the mother with the possible exception of methylene blue, where poor pregnancy weight gain was noted. This compound yielded, incidentally, the highest percentage of resorptions (38.0%). Of particular interest is the observation that two of the widely used commercial antioxidants, butylated hydroxytolulene (BHT) and butylated hydroxyanisole (BHA), as well as the antioxidant vitamins alpha and gamma tocopherol, at 200 mg level, and 2,5-di-tert-amylhydroquinone gave the lowest percentage of resorptions.

Data on the distribution of resorption sites in all groups revealed that 47.6% of all resorptions were located in the cranial third of the horns, 20.6% in middle third and 31.8% in the caudal third. Most of the sites seen in this cranial region were small inconspicuous cicatrixic plaques suggestive of an early embryonic death.

Using the classification of Walker and Wirtschafter ('56) most of the resorptions, regardless of dietary supplement, belonged either to grade 3, representing complete resorption of the embryo but with small placenta still present, or grade 4, which are cicatrized uterine plaques marking previous placental sites. Grade 2, consisting of small macerated recognizable fetuses, were seldom encountered. The number of fetuses falling into grade 1, e.g., dead, but fully formed, could not be determined since many of our fetuses were killed by anoxic treatment of the mother.

DISCUSSION

Since our observations dealt with the identification of placental sites *in situ*, the question might be raised if identifiable plaques form at all implantation sites. That such scars do form where embryos have been resorbed is well documented by the work on rats of Davis and Emlen ('48). They reported more scars present than young born, the excess being ascribed to resorbed embryos. They also noted that scars do not occur spontaneously in nonpregnant rats. Moreover, these authors and Conaway ('55) reported that the resorption sites may remain identifiable for at least a year in the rat, and Deno ('41) made the same observation on the mouse. Therefore our use of first gestation animals avoided the possible error of counting sites of earlier pregnancies and ascribing them to the gestation under study.

The range of reported resorptions in normal rats by other investigators (11.8–6.5%) (table 2) may be due to seasonal variations in the fertility of the animals. It is generally accepted by rat breeders that fertility is lowest in winter and highest in summer (Main, '39). Since our data was collected throughout the year under controlled environmental conditions, we were able to separate our findings on a seasonal basis. We noted a reduction in the percentage of resorptions during the spring and summer (8.1%) and a marked increase (20.7%) during the winter and fall. Frazer ('55) using Wistar albino rats reported that 31% of all litters had some prenatal mortality on a yearly average, but that it was lowest in July-September (17%) and highest from October to December (86%). Other workers failed to present their prenatal mortality data on a seasonal basis, therefore an accurate comparison of animal groups is difficult, if not impossible.

Another variable influencing the percentage of resorptions in the rat may be the strain difference. Data on 6 different strains, including the wild rat, are summarized in table 2 and, with the exception of our WR-CF animals (10.6%) and Mason's O.M.–Mc. rats (11.8%) the 4 remaining groups agree rather closely on resorption percentages (6.6–8.7%). Yet the strain of the animal may be important, for the findings of Fekete ('47) in the mouse that fertilized ova transplanted to different strains of mice have a significantly better chance of survival in one strain than another, implies that one strain provides a better environment for implantation and development of the ovum. Hollander and Strong ('50) also noted significant differences in fetal mortality among various strains of mice. It is certainly possible that in the rat also, strain differences may exist which could

TABLE 2

Prenatal mortality in mammals

Animal	Litters	Implantations	Normal fetuses	Resorptions	Corpora lutea	Total prenatal mortality	Reference
				%			
Striped hamster	29	193	119	38.3			Fortuyn, '29
Stoat	12	108	103	4.6	135	23.7	Deanesley, '35
Grey squirrel	9		27		30	10.0	Deanesley and Parkes, '33
Sheep	80	109	101	7.2	116	12.9	Hammond, '21
Pig	22	316	267	15.5	396	32.5	Hammond, '21
Pig[1]	495	3528	3485	1.2	4480	22.2	Corner, '23
Pig	102	1117	1028	8.0	1396	26.1	Crew, '25
Rabbit (wild)	68	350	340	2.9	390	12.8	Hammond, '21
Rabbit (wild)	1819		8877		10574	16.0	Brambell, '48
Rabbit (wild)	2179	11363			12551	9.4	Brambell and Mills, '47
Rabbit (wild)	1834	9658	8927	7.6			Brambell and Mills, '48
Rabbit (tame)	56	441	391	11.3	578	32.3	Hammond, '21
Mouse	1080	7967	6672	16.3			Hollander and Strong, '50
Mouse[2]	500	3175	2930	7.7			Danforth and deAberle, '28
Rat (wild brown)	131	1238	1135	8.3	1420	20.0	Perry, '45
Rat (OM-Mc)[3]	50	492	434	11.8			Mason
Rat (Wistar)	25	226	211	6.6			MacFarlane et al., '57
Rat (Wistar)[4]	6	57	52	8.7			Wilson, '54
Rat (Long-Evans)	156		998		1498 (ova count)	33.4	Long and Evans, '22
Rat (Long-Evans)[5]	42	447	410	8.3			Nelson et al., '52
Rat (Holtzman)	35	371	347	6.5			Cheng and Thomas, '53
Rat (WR-CF)[6]	126	1430	1278	10.6			Telford, Woodruff, Linford

[1] Omitted from this data were 13 litters with all implanted embryos resorbing.
[2] From data on 500 pregnancies during last third of gestation as compared to 500 litters 1–3 days old.
[3] OM-Mc designates a mixed strain derived from Osborn-Mendel and McCollum piebald strains (data from personal communication).
[4] Reports present resorptions about 4–5% (personal communication).
[5] Pteroylglutamic acid control animals. Current resorptions in Long-Evans rats about 5% (personal communication).
[6] WR-CF indicates original Carworth Farms stock but inbred at Walter Reed Army Research Center.

account for the fairly wide range of prenatal death rates.

Our analysis of the location of resorption sites in the various regions of the uterine horn revealed that 47.6% of all resorptions were found in the cranial third of the horn, with 20.7% in middle third and 31.8% in caudal third, confirming our impression that the cephalic tapered extremity of the horn was, perhaps, an unfavorable location for the developing embryo. This concept is supported by the observation of Fawcett, Wislocki and Waldo ('47) that the overcrowding by the developing mouse ovum in the anterior chamber of the eye inhibited the growth of adjacent implanted ova. However, Frazer's ('55) data on the rat does not favor this idea, for he demonstrated that the percentage resorption was the same in both horns even though different number of ova were implanted in the different horns. Furthermore, the overcrowding of a mouse uterine horn with an unusually larger number of implanted ova did not influence adversely the resorption rate, as shown by Hollander and Strong ('50).

Another variable influencing fetal loss is the maternal age or weight. Frazer ('55) noted in two strains of rats that the heavier the mother, the higher resorption rate. Similar findings had been reported earlier by Hollander and Strong ('50). All of our animals were standardized at approximately 200 gm giving us uniform test animals thus eliminating the possible discrepancies due to age or weight.

Combining the data in table 2 for all groups of the same species, reveals that the tame rat values fall roughly in the middle of the other mammalian data. For example, the tame rat data show that 9.2% of all implantations end in resorptions. Those mammals with a lower percentage of resorptions are, pig (3.7%), stoat (4.6%), sheep (7.2%), wild rabbit (7.3%) and wild rat (8.3%). Those animals suffering on the average greater fetal loss than the rat are, tame rabbit (11.3%), mouse (15.9%) and wild hamster (38.3%). While fetal wastage is probably common to all mammals considerable species variation does exist.

Basic to our study of the value of antioxidants in the reduction of fetal mortality is the question of their mode of action and whether the site of action of the compound is on the fetal or maternal side of the placenta or both. Most of the agents reported here have been previously used in animal experimentation (Bunnell et al., '55; Schwarz, '58; Mertz and Schwarz, '58; Dam et al., '48, '51). From the results of these workers we assumed that the compound could be assimilated into the blood stream and may exert some influence on the developing placenta and might affect fetal growth. Evidence of placental transfer, however, is available only on a limited number of the tested agents, such as glucose (Huggett, '54), α-tocopherol (Neuweiler, '49), thiamine chloride (Slobody, Willner and Mestern, '49), ascorbic acid (Hamil et al., '47) and phenothiazine (Behn, Frahm and Fretwurst, '56). The rate of placental transfer of fat and water soluble vitamins is quite different, with the lipid soluble vitamins being much slower. In fact in the rat the transfer may be so slow that the cord blood content of vitamin A (Lund and Kimble, '43) or E (Straumfjord and Quaife, '46) may, under certain circumstances, be less than in maternal blood. In the rat Mason and Bryan ('40) reported only limited placental transfer of vitamin E derived from a wheat germ oil concentrate. Yet if 250 mg tocopherol is administered to women 4–8 hours before term, the vitamin E level in the cord is raised significantly (Varangot, Chailley and Rieux, '44). By contrast the water soluble vitamins, thiamine chloride (Slobody, Benson and Mestern, '46) and ascorbic acid (Manahan and Eastman, '38; Chow and Okuda, '60) showed consistently higher concentrations in cord than maternal blood, suggesting even perhaps a concentration of the vitamins by the placenta.

There is a great deal of evidence that in the rat vitamin E is essential for normal fetal development, for example, Cheng, Chang and Bairnson ('57) showed that in vitamin E deficiency 100% resorption of embryos occurred, while no resorption occurred in pregnant rats receiving daily 2 mg d,l-α-tocopherol acetate. Our data showed only 4.1% resorption in rats receiving 10 mg *per os* for 20 days during gestation as compared to 10.6% for all

normal untreated controls. Daily 5 mg dosages of α-tocopherol administered to another group of pregnant animals had no beneficial effect on resorption rate, in fact some negative response was noted (14% resorptions).

In addition to high dosages of α- and γ-tocopherol our data suggest some beneficial effects on resorption rate were obtained from 2,5-di-tert-amylhydroquinone, butylated hydroxytolulene (BHT), butylated hydroxyanisole (BHA), 2,6-di-tert-butyl-4-methylphenol (DBMP), thiodipropionic acid, 1,2-di-hydro-2,2,4-trimethyl-6-ethoxyquinoline (Santoquin), 6-phenyl-1, 2-dihydro-2,2,4-trimethylquinoline (Santoflex B), nordihydroguaiaretic acid (NDGA); while methylene blue, hydroquinone, paraminobenzoic acid (PABA), n propyl gallate, 2,2-methylene bis (4-ethyl-6-tertiary butyl phenol), phenothiazine and N, N-diphenyl-p-phenylenediamine (DPPD) all substantially increase resorption rates.

One is impressed from the data summarized in table 2 that the total prenatal mortality in mammals is much higher than might be suspected from the resorption data alone. Brambell ('48) contends in the rabbit, "that the loss between ovulation and full term is not less than 43% of ova ovulated and may be, and indeed probably is, substantially heavier." Data on total wastage in the rat is scanty, limited to the early work of Long and Evans ('22) and later Perry ('45) on the wild rat. These workers reported respectively 33.4% and 20.0% total prenatal mortality while resorption rate was 8.3% for the two strains of rats.

Other mammals follow a similar trend, ranging from an elevenfold increase in total mortality over resorptions in pig (Corner, '23) to less than a twofold increase in sheep (Hammond, '21). Such observations certainly provide a challenge to animal research workers to seek out the causative factors for such an enormous wastage. These factors are legion, but include such environmental disturbances as abnormalities of blood supply of placenta, an unfavorable implantation site, faulty development of the decidua, abnormal environmental temperature and others. Maternal influences may adversely affect normal implantation and development, such as, inanition, avitaminoses, blood dyscrasias, endocrine disorders, infections and the presence of unphysiologic compounds in the blood such as some of the antioxidants herein reported.

SUMMARY AND CONCLUSIONS

1. In 126 untreated normal pregnant rats 40.8% had one or more resorptions and 10.6% of all implantations terminated in resorptions.

2. The cranial third of the uterine horn contained 47.6% of all resorptions, while the middle third held only 20.6% of the total resorptions.

3. The percentage resorptions in the tame rat falls intermediate among the other mammalian groups.

4. Of the maternally administered compounds, the tocopherols (α and γ), BHT, BHA, amylhydroquinone and DBMP appear to have definite prophylactic value in reducing the frequency of resorptions in the rat, while methylene blue, hydroquinone and paraminobenzoic acid markedly increase resorption rates.

5. Possible extrinsic and intrinsic factors that may influence prenatal mortality in the rat are discussed.

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The Influence of Propyl Gallate and α-Tocopherol on the Survival Time of Rats during Fasting*

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Abstract—A toxicological investigation was carried out in order to repeat and extend the findings of Bukhman (*Pop. Pitan.* 1962, **21**, 68) concerning the toxicity of propyl gallate in fasting rats. Rats were given a diet containing 0·002 or 0·004% propyl gallate or α-tocopherol. After 13 wk some of the animals were sacrificed, while the remaining rats were placed on a partial starvation diet until death. The survival time was recorded. Chemical determinations of water, protein and fat content were made on the sacrificed animals as well as on those that died. No difference in survival time or in protein and fat utilization was found between the control groups and the groups which received propyl gallate or α-tocopherol. The results of Bukhman could not be confirmed.

INTRODUCTION

Propyl, octyl and dodecyl gallates are extensively used as antioxidants in fats. On the basis of detailed toxicity studies, an acceptable daily intake for man of 0–0·5 mg gallates/kg body weight was estimated by the Joint FAO/WHO Expert Committee on Food Additives (1965).

Our attention has been directed to an unconventional approach to toxicity testing. In a study by Bukhman (1962) rats were fed for 13 wk on a diet containing 15% fat to which was added 0·02% propyl gallate, a concentration equivalent to 0·003% of the total diet. After this period, the rats were placed on a partial starvation diet until death. At the end of the first period, several animals were sacrificed and the fat and protein content of their bodies determined. Animals which died during the fasting period were treated in a similar manner. From Bukhman's results it appeared that the average survival time of the animals was considerably reduced by the propyl gallate, from 26·8 days in the control group to 14·7 days in the experimental group. According to the author, the control animals utilized their body fat and proteins better than the animals which had been fed propyl gallate. It was postulated that the oxidation processes in the organism were inhibited by the antioxidant and that this resulted in an incomplete utilization of the body fat and protein. To study the value of this type of toxicological investigation, a comparative investigation has now been carried out, in which propyl gallate and α-tocopherol were tested.

*Publication of this paper has been agreed upon because the work upon which it is based arose from the following comment by the Joint FAO/WHO Expert Committee on Food Additives (1965, p. 25): ". . . The results of the experiments involving partial starvation are of great interest and indicate a higher toxicity of propyl gallate than the other studies. Evaluation of these experiments is difficult, however, in the absence of comparable studies with other food additives. . . ." By demonstrating that the claims made by Bukhman could not be substantiated, the authors have shown that cruel and unphysiological experiments which involve starving animals to death have little scientific merit and absolutely no justification as a method of safety evaluation—Editor.

EXPERIMENTAL

Materials. Propyl gallate was obtained from Chemische Fabriek "Naarden", Naarden, The Netherlands, and α-tocopherol from Hoffmann-La Roche, Basel, Switzerland. Fresh lard was obtained weekly from the slaughterhouse and melted down.

Animals and diets. Six groups of Wistar rats (initial body weight 40–60 g) were used in the experiment. The control lard group consisted of 30 male and 30 female rats which received 15% lard in a diet containing 59% whole-wheat flour, 25% skim-milk powder and 1% salt mixture. Four other groups, each consisting of 15 male and 15 female rats received the same diet to which 0·002 or 0·004% propyl gallate or α-tocopherol was added. In Bukhman's experiment only one dietary level of propyl gallate (0·003%) was used. In the present study, an additional control group containing 15 male and 15 female rats was included for purposes of comparison. These animals (control WMS group) received a standard diet consisting of 66% whole-wheat flour, 33% whole-milk powder and 1% salt.

Methods. For histopathological studies, organs and tissues were fixed in 4% formalin. Paraffin sections were stained with haematoxylin and eosin; frozen sections were used for Oil Red O staining. The test of Wilcoxon (1945) was used for statistical calculations, unless otherwise stated.

Feeding study and analyses. During the first 13 wk, tapwater and diets were given *ad lib.* and growth and food intake were recorded. At the end of this period, 10 males and 10 females from the control lard group and 5 males and 5 females from each of the other groups were sacrificed. The liver, kidneys, heart, spleen, thymus, adrenals, thyroid, pituitary, pancreas, brain, uterus, ovaries, testes and prostate were weighed. Histopathological studies of the organs of the rats of both control groups and of the groups on 0·004% propyl gallate and 0·004% tocopherol were carried out. Blood was taken for haemoglobin estimation and erythrocyte and leucocyte counts. The protein content of the liver was determined. Organs, head, tail, feet and skin were removed and the rest of the carcass was packed in a sheet of plastic and frozen at −20°C. The frozen carcass was ground in a meat mincer and weighed. A small part was taken out for a protein determination and the rest was dried to constant weight at a temperature of about 90°C, in order to determine the water content. A part of the ground dried carcass was used for fat determination by petroleum ether extraction in a Soxhlet apparatus. The protein content of the liver was estimated after homogenization of about 60 mg in water and that of the ground carcass after homogenization of 60 mg in N-NaOH solution. The homogenates were subjected to protein determination by the method of Lowry, Rosebrough, Farr & Randall (1951).

Starvation and analyses. After 13 wk, the remaining animals were placed in individual cages on a fat-free diet having 20% of the calorific value of the normal mean daily food intake for male rats. Each animal received 3·5 g food/day, and the animals were maintained on this diet until they died. The survival time of each rat was recorded and the same chemical determinations were carried out as on the sacrificed rats.

Recovery period. After 9 wk on the partial starvation diet (22 wk from the beginning of the experiment), the surviving animals were fed the control WMS diet *ad lib.* to detect any eventual difference in recovery between the groups. These animals were sacrificed 2 or 4 wk later and haematological and chemical analyses were carried out.

RESULTS

Growth, food intake, haematology and organ weights

During the first 13 wk, growth and food intake of the animals receiving antioxidants did

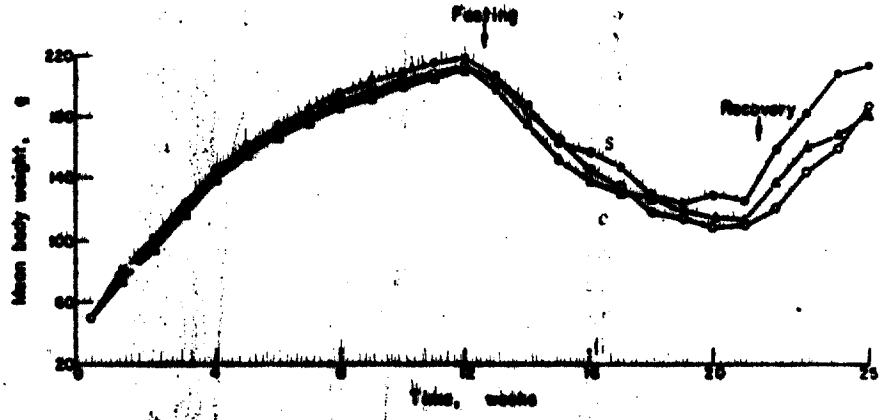

FIG. 1. Growth curves of female rats during a feeding period of 13 wk, a subsequent partial starvation period of 9 wk and a recovery period. Numbers of animals: wk 1–13, at least 30; wk 13–22, 20–3; wk 22–26, 3 or 4. ○, control lard groups; ●, 0·002 + 0·004 % propyl gallate groups; ▲, 0·002 + 0·004 % α-tocopherol groups.

not differ significantly from those of the control groups. In Fig. 1 the growth curves of the female control rats and female rats on propyl gallate and on α-tocopherol are given. It is clear that these animals did not show any difference in growth. The same was the case for the male animals. The partial starvation diet, starting after 13 wk caused a rapid weight loss in all groups. After a 9-wk fasting period, the 1 male and 13 females still alive were placed on the control WMS diet. The body weight of these rats recovered rapidly. No significant differences in the blood composition or in organ weights relative to body weight or heart weight were found that could be ascribed to the administration of the antioxidants.

Survival time after fasting

A striking difference in survival time was found between male and female rats. Since there was no difference between the two dose levels of antioxidants, data from the groups receiving 0·002 and 0·004 % propyl gallate were combined for statistical calculations. The same was done for the groups receiving 0·002 and 0·004 % α-tocopherol. The difference in survival time between males and females was statistically significant for the control and the tocopherol groups ($P < 0·05$) and for the gallate groups ($P < 0·01$). In Table 1 the cumulative percentage mortality after varying periods on the partial starvation diet is given for male and female animals.

Table 1. *Effect of prior administration of antioxidants on cumulative percentage mortality of rats during a period of partial starvation*

Diet	Cumulative mortality (%) at day*						
	15	20	25	30	40	50	65
Males							
Control lard	5	25	55	80	95	100	100
Gallate (both levels)	14	37	67	81	86	90	95
Tocopherol (both levels)	15	40	70	95	95	100	100
Control WMS	0	50	70	80	100	100	100
Females							
Control lard	5	35	45	65	65	85	85
Gallate (both levels)	0	26	42	42	42	74	84
Tocopherol (both levels)	5	30	35	60	75	85	85
Control WMS	10	30	50	50	60	60	60

*From beginning of starvation period.

The data given in Table 1 do not show a clear difference between the rats of the control groups and the rats that had received propyl gallate or α-tocopherol in their diet. Most of the animals died in the first 15–20 days after the beginning of the fasting period, but there was a large variation. After a 9-wk fasting period, 14 animals were still alive and it was therefore difficult to calculate a mean survival time. An attempt was made to do this by giving the surviving animals an arbitrary survival time of 65 days, since from that time on they were placed on a normal diet. The results of this calculation are presented in Table 2 and show no statistically significant difference between the groups for either sex.

Table 2. *Effect of antioxidants on "mean survival time" of rats during fasting*

Diet	Males		Females	
	Death rate	Mean survival time (days)	Death rate	Mean survival time (days)
Control lard	20/20	26·0	17/20	32·6
Gallate, 0·002%	11/11	21·2	8/9	40·8
Gallate, 0·004%	9/10	28·1	8/10	38·0
Tocopherol, 0·002%	10/10	24·3	8/10	37·3
Tocopherol, 0·004%	10/10	20·9	9/10	27·2
Control WMS	10/10	22·6	6/10	40·0

Chemical analyses

Animals sacrificed after 13 wk. The water, fat and protein content of the carcasses as well as the protein content of the liver are summarized in Table 3. The results, except in the case of the liver, are expressed as percentages of the minced carcass.

Animals dying during fasting. In all rats that died during the fasting period the same chemical analyses were carried out as in the rats that were sacrificed after the 13-wk feeding period. The results are given in Table 3. When these results are compared with those of the rats that were sacrificed before the fasting period, a statistically significant difference ($P < 0{\cdot}001$ in all cases) in body weight and water and fat content of the animals is apparent. The decrease in body weight was considerable, averaging 43·9% for the males and 42·5% for the females, and became more pronounced with longer survival. With Spearman's rank correlation test (Jonge, 1963) a significant negative correlation ($P < 0{\cdot}01$) was found between the survival time and the body weight for both male and female rats when data for all groups were combined.

Animals sacrificed after recovery. One male and 13 female rats still alive after the 9-wk fasting period were placed on the control WMS diet. From Fig. 1 it is clear that the body weight recovered rapidly. Half of the animals were sacrificed after 2 wk and the rest after 4 wk. The same chemical analyses were carried out as in the other groups of rats. Since there appeared to be no difference (except in body weight) between the animals sacrificed after 2 or 4 wk, the results have been combined in Table 3.

These results show that a normal food intake for 2 wk is sufficient to restore the water and fat content of the rats to the same level as before the fasting period. Only the animals of the control WMS group had a tendency to a higher fat and a lower water content than before. The weight gain after 2 wk was 69% for the control WMS group and averaged 42% for the other groups. After 4 wk these figures had increased to 83% and 74% respectively. The organ weights and haematological findings did not show any deviations.

Table 3. *Water, fat and protein content of the rats at various stages of the test*

Diet	No. of animals	Body weight (g)	Carcass			Liver
			Water (%)	Fat (%)	Protein (%)	Protein (%)
Males		Animals sacrificed after 13 wk				
Control lard	10	280	67·3	6·21	19·7	22·8
Gallate, 0·002%	5	284	67·4	4·94	21·0	23·9
Gallate, 0·004%	4	301	67·6	5·15	20·0	21·4
Tocopherol, 0·002%	4	289	68·0	5·75	19·5	20·5
Tocopherol, 0·004%	5	285	67·4	6·05	19·7	20·7
Control WMS	5	302	67·4	6·55	19·3	20·9
Females						
Control lard	10	194	67·2	7·62	19·9	22·2
Gallate, 0·002%	5	205	65·1	7·63	19·7	22·1
Gallate, 0·004%	5	206	64·7	9·09	21·0	22·5
Tocopherol, 0·002%	5	206	64·5	8·79	18·7	20·6
Tocopherol, 0·004%	5	196	66·0	7·62	19·6	21·0
Control WMS	5	199	65·5	7·94	18·4	21·4
Males		Animals dying during fasting				
Control lard	19	161	70·8	0·34	17·6	20·8
Gallate, 0·002%	11	175	71·1	0·29	18·0	19·7
Gallate, 0·004%	9	150	71·1	0·33	18·1	19·8
Tocopherol, 0·002%	10	152	70·4	0·34	19·4	19·9
Tocopherol, 0·004%	10	159	70·4	0·33	18·7	21·1
Control WMS	10	171	70·3	0·32	19·1	21·6
Females						
Control lard	17	122	69·9	0·38	18·3	20·8
Gallate, 0·002%	8	107	70·2	0·48	17·2	21·5
Gallate, 0·004%	8	116	69·6	0·41	18·6	21·0
Tocopherol, 0·002%	8	111	70·0	0·47	17·8	21·7
Tocopherol, 0·004%	9	117	70·4	0·44	18·3	21·6
Control WMS	6	116	69·4	0·38	19·6	21·4
		Animals sacrificed after recovery				
Control lard	3	—	65·9	7·48	18·0	21·1
Gallate (both levels)	4	—	65·3	9·51	19·0	21·0
Tocopherol (both levels)	3	—	66·1	7·38	19·6	20·8
Control WMS	4	—	63·0	12·79	20·4	20·0

In any given period, no statistically significant difference was found between the groups for any of the determinations made.

Histopathology

Extensive histopathological examinations were carried out on all the organs and tissues which were weighed, except the brain and the thymus. The digestive tract and the bladder were also studied. Because many of the fasting animals died during the night, a marked autolysis was found in the tissues of these animals, especially in the digestive tract. All animals which died during the starvation period were extremely cachectic and most of them showed oedema.

Neither the sacrificed animals, nor those which died after fasting showed any pathological changes which could be attributed to the treatment with antioxidants.

DISCUSSION

This experiment was carried out in order to repeat and extend a toxicity study with propyl gallate carried out by Bukhuizen (1962). In his experiments, animals given propyl gallate in their diet showed a reduction in weight gain and delayed development. During partial fasting the rats receiving propyl gallate failed to utilize their body fat and protein adequately and succumbed at an earlier date. Bukhuizen found a mean survival time of 14·7 days for these groups and of 20·8 days for the groups not receiving propyl gallate. He used only male animals.

In our experiment, rats of both sexes were used. It appeared that the survival time for female animals was longer than that for male animals. In fact there were 13 females and 1 male still alive 9 wk after the beginning of the starvation period. When these animals were given an arbitrary survival time of 65 days, an average of 23·9 days was found for the male animals of all groups and 36·1 days for the females. No difference in survival time was observed between the control and test groups. The longer survival of the female animals can probably be explained by the fact that the starvation diet (20% of the calorific value of the normal daily food intake) was based on the food consumption of the male animals. The females were given the same quantity of food, although they normally need less food than the males.

The determination of the water, fat and protein content of the carcasses did not show any differences between control and treated animals. Animals that died during the partial starvation period had a significantly higher water content than the animals sacrificed after the 13-wk feeding period. This was possibly due to starvation oedema. The fat content of the animals that died was significantly lower than that of the sacrificed animals. There was no difference in fat content between the animals that died 10–15 days after the beginning of the starvation period and those dying after more than 40 days starvation. Obviously the fat was utilized quite rapidly at the beginning of this period in spite of the administered antioxidant, but a very small amount, less than 0·5%, always remained.

The protein content of the rats showed a tendency to be lower in the animals dying during the starvation period than in the sacrificed animals, especially when they survived more than 40 days, but the difference was not significant. The protein content of the liver was not affected by fasting.

In contrast to Bukhman's postulation, our biochemical analyses did not show that rats fed diets containing propyl gallate use their fat and protein resources less effectively than the controls. In no case was there a significant difference in fat or protein content between the groups. Possibly this can be explained by the fact that Bukhman expressed his results in terms of absolute amounts of protein and fat and not as percentages of the body weight.

We have found a strongly negative correlation between survival time and body weight. In Bukhman's paper no body weights were given, but since our results indicated a relatively constant protein content of 20% for all rats, we can calculate from his figures the body weight before fasting and when the animals died. In this way we have also calculated the weight loss of his animals when they died, and found for the groups without propyl gallate a mean value of 33·5% and for the groups receiving fats containing 0·02% propyl gallate a mean value of 21·9%.

In our investigation a loss in body weight of 43·9% for the males and 42·5% for the females was found. It seems that in our test, animals consuming antioxidants utilized their protein more effectively than the animals on a gallate-free diet in Bukhman's study. Since no

pathological findings were reported by Bukhman, it is not known why the animals with propyl gallate in their fat died when they had lost only 21·9% of their body weight.

Our conclusions on propyl gallate also apply to α-tocopherol, since, as the tables and figure show, the results for the two compounds are closely comparable.

Since we could not confirm Bukhman's results, it has been concluded that this type of study makes no useful contribution to the toxicological investigation of antioxidants or other substances.

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Influence du gallate de propyle et de l'α-tocophérol sur la durée de survie des rats pendant le jeûne

Résumé—Une étude toxicologique a été conduite afin de reproduire et de compléter les données de Bukhman (*Vop. Pitan.*, 1962, 21, 68) en ce qui concerne la toxicité du gallate de propyle chez les rats soumis au jeûne. Des rats ont reçu un régime contenant 0,002 à 0,004% de gallate de propyle ou d'α-tocophérol. Au bout de 13 semaines, certains de ces animaux ont été sacrifiés, les autres étant mis sous un régime de famine partielle jusqu'à leur mort. On a relevé la durée de leur survie. On a procédé à des dosages de l'eau, des protéines et des lipides aussi bien chez les animaux sacrifiés que chez ceux qui sont morts. On n'a trouvé aucune différence quant à la durée de survie ou à l'utilisation des protéines et des lipides entre le groupe témoin et celui auquel était administré le gallate de propyle ou l'α-tocophérol. On n'a pas pu confirmer les résultats qu'avait obtenus Bukhman.

Der Einfluss von Propylgallat und α-Tocopherol auf die Überlebenszeit von Ratten während des Fastens

Zusammenfassung—Es wurde eine toxikologische Untersuchung durchgeführt, um die Ergebnisse von Bukhman (*Vop. Pitan.* 1962, 21, 68) hinsichtlich der Toxizität von Propylgallat in fastenden Ratten zu wiederholen und zu ergänzen. Ratten erhielten Futter mit einem Gehalt von 0,002 oder 0,004% Propylgallat oder α-Tocopherol. Nach 13 Wochen wurden einige Tiere getötet, während die verbleibenden Ratten ein zum Tode führendes unzureichendes Futter erhielten. Die Überlebenszeit wurde bestimmt. Chemische Bestimmungen des Wasser-, Protein- und Fettgehalts wurden an den getöteten und an den verendeten Tieren durchgeführt. Hinsichtlich der Überlebenszeit oder der Protein- und Fettverwertung wurde kein Unterschied zwischen den Kontrollgruppen und den Gruppen festgestellt, die Propylgallat oder α-Tocopherol erhielten. Die Ergebnisse von Bukhman konnten nicht bestätigt werden.

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Effect of Grisin and Vitamin A and E on Natural Immunity in Chickens

by

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Notwithstanding the large number of studies devoted to the investigation of antibiotics and vitamins, still insufficiently explained is the effect of these preparations on the physiological reactivity of a macroorganism, its specific and nonspecific protection mechanisms (1).

There are works showing that antibiotics and vitamins are capable of positively influencing immunogenesis in the organism. Studies (2-7) established that the antibiotics penicillin, streptomycin, levomycetin, sanazin, chlortetracycline and others during their introduction to immunized experimental animals increased their titer of antibodies and blood complement and stimulated phagocytosis.

We observed a significant decrease of mortality of mice under the effects of streptomycetin (8).

Z. V. Yermol'yeva (9) points out that antibiotics, besides specific action on microbes, have positive effects on macroorganisms and can have an influence on the formation of an immunity during certain illnesses.

A positive effect of fodder antibiotics and vitamins on immunogenesis has been established (10-12).

A change in electrophoretic availability of ...(illeg.) blood serum under the influence of antibiotics has been established (13).

Works (14-17) occasioned much interest, confirmed in the experiment of the possibility of immunobiological reorganization of the organism under the effects of the introduction of the antibiotics tetracycline, penicillin, streptomycin, terramycin, neomycin, and others.

Together with these studies there are studies showing that the introduction of antibiotics during immunization or medical treatment of infectious diseases does not effect the protective immunobiological reactions of the organisms, or depress them (18-21).

In this way the results of numerous studies on the effect of antibiotics on immunogenesis are analyzed; it is possible to arrive at the conclusion that the introduction of antibiotics undoubtedly has an effect on immunobiological characteristics of the organism, the depression of it or its stimulation. The degree of this effect

is determined probably either by the various methods of research and the formulation of the experiments, by the dosages of antibiotics and the duration of their introduction, by the scheme of medical treatment of infectious diseases, or by the characteristics of the pathogenesis and immunogenesis (18).

It is necessary to note that the majority of the above quoted studies were carried out either in the course of the infection process or in the preceding immunization. Questions concerning the effect of antibiotics and vitamins on the factors of natural (non-specific) immunity in the organism, are illuminated only in works (22, 23).

There are studies in this direction of great significance as it is known that the natural resistivity stipulates an effective specific immunoprophylactic and determines the resistance of the organism to infectious diseases (24).

In our three-year experiments (1962-1964) on chicks, the following antibiotics were used: grisin (crystalline preparation), kormogrizein (fodder form of the antibiotic grisin, active and "low-active"), vitaminized chlortetracycline fodder (BKV)-control to kormogrizein and vitamin A and E (tocopherol).

The antibiotic grisin as is known was obtained by a group of scientists under the direction of Corresponding Member of the Academy of Sciences of the USSR professor N. A. Krasil'nikov in the Institute of Microbiology of the Academy of Sciences of the USSR, from the producer Act. griseus strain 15 (25). Subsequently in 1962 from the cultured fluid producer of the antibiotic grisin, N. A. Krasil'-nikov, Ya. I. Rautenshteyn, A. I. Korenyako, N. I. Nikitin and others obtained the fodder form of this antibiotic -kormogrizein (26). Studies on the analysis of kormogrizein showed that that preparation seems to be a stimulant of growth and development of the organism of young animals and birds and serves as an effective medico-prophylactic agent for the medical treatment of a series of infectious diseases (27-30).

In our experiments on the background of growth-stimulating effects, the effect of the antibiotics grisin, kormogrizein and vitamins on the factors of natural immunity of chicks was studied. In the experiment were 2220 chicks of the White Russian breed, 1260 from 25 to 30 days old and 960 from 3 to 4 days old (experiments on the survival of chicks in the first days of life). All of the experimental chicks were broken down into their homologous groups and contained in a cage with 20 to 25 specimens (25-30 days old) or 60 specimens (3-4 days old).

Each series of experiments lasted 25 days. In that length of time daily one time per day in their fodder the within their homologous groups received a supplement of the preparations being studied the antibiotic grisin (previously dissolved in water) in dosages of 1-1.6 mg per chick, active (2000 units of activity of Staph. aureus strain 209), and kormogrizein "low-active" (27 units of activity) in dosages of 3-5 g per 1 kg of fodder, concentration of lipid-soluble vitamins- A (526,000 nmu per gram, in dosages of 0.02

ml as water emulsion) and E (10 mg of tocopherol in 1 g of preparation, in dosages of 0.1 ml per each chick as water emulsion). Two groups of chicks were taken as our controls. One of these in their fodder received the supplement vitaminized chlortetracycline fodder (control to the kormogrizein, in dosage as the prescribed instructions). The second (control) group of chickens supported themselves on a regular fodder ration without supplemental antibiotics or vitamins.

They demonstrated a natural immunity which was studied by the following method. From the axillary vein of the experimental chicks (25-30 days old) blood was drawn (up until their fortification with antibiotics and vitamins, and then on the eighth, 15th, and 25th days in the tests for activity) for the formulation of the reaction of agglutination (they determined for themselves the titer of normal antibody-agglutinin, dilution of serum- 1:4 - 1:20)with the antigen pullorin and the poultry typhus and a determination of the activity of the blood complement (titer 0.15-0.45 ml). For the determination of the phagocytic activity of the leukocytes, the number of phagocytes in a blood smear were calculated, dyed by the Romanov-Gimza method.

The growth-stimulating effect, practicable from the calculation of the applications of the studied antibiotics and vitamins, determined the method of periodic weighing of the experimental chicks, simultaneously with the taking of the specimen of blood. The degree of survival of 3 to 4 day old chicks (of natural loss) was determined by the method of daily collective record-keeping of the livestock each experimental group.

Results of the research conducted are presented in Figures 1 through 3.

From the presented drawings it can be seen that the studied antibiotics grisin and active kormogrizein on the background of growth-stimulating effects (weight increase in the chicks resulting from the grisin was on the average 30%, and resulting from kormogrizein- 31-57% higher than the controls) stimulated the production of normal antibody-agglutinins (titer of antibodies increased 5 to 10 times in comparison with the controls), significantly increased the activity of the blood complement (more than two times higher than the controls), and stimulated the phagocytic activity of the chicks' leukocytes.

"Low-active" kormogrizein, biomycin fodder, vitaminized chlortetracycline fodder, vitamins A and E, together with the growth-stimulating effect also stimulate production of factors of normal immunity in chicks. However, these preparations possess less expressed capacity to act on the properties of natural immunity of chicks, than during the use of the antibiotics grisin and active kormogrizein. It may be especially clear to follow these indices in the 15th day of the experiment.

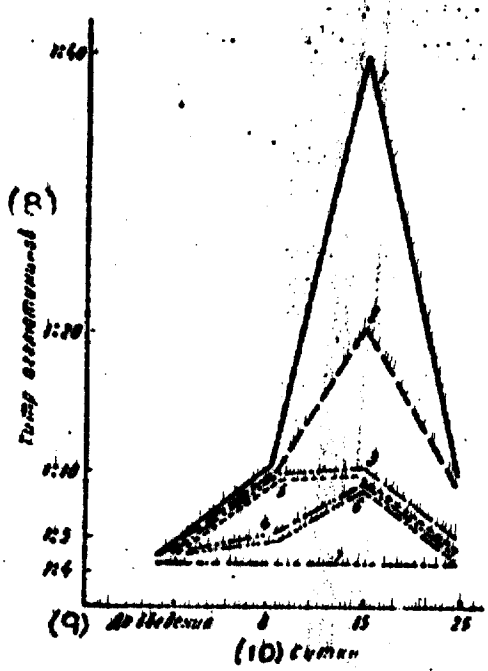

Fig. 1. Reaction of agglutination in chicks during the addition of grisin, kormogrizein and vitamins.
1- Grisin; 2- active kormogrizein; 3- "low-active" kormogrizein; 4- fodder biomycin; 5- vitamin A; 6- vitamin E; 7- control (normal fodder ration); 8- titer agglutinin; 9- until addition of supplements; 10- days.

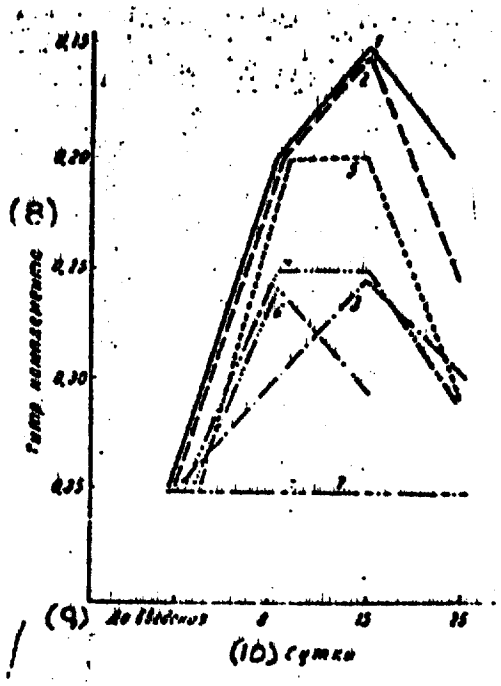

Fig. 2. Activity of blood complement in chicks during the addition of grisin, kormogrizein and vitamins.
1-7- Denotation same as for Fig. 1; 8- titer complement; 9- until addition of supplements; 10- days.

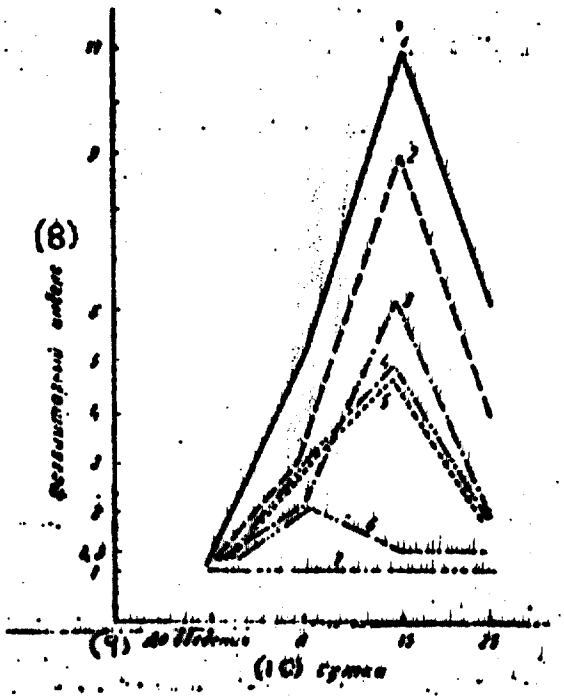

Fig. 3. Phagocytic activity in chicks during addition of grisin, kormogrizein and vitamins.
1-7- Denotation same as for Fig. 1; 8- phagocytic index; 9- until addition of supplements; 10- days.

Analysis of the results of the studies carried out by the method of variation statistics (31) confirmed the authenticity of the data obtained. The antibiotics grisin and kormogrizein in comparison with the vitamins and the controls proved to be a greater stimulant of the production of the standard factors of natural immunity in chicks and give a reliably greater growth-stimulating effect ($P < 0.05$ or $> 95.5\%$).

As our experiments have shown, the use of fodder antibiotics and vitamins increases the survival rate in chicks (reduces natural loss) in the first fifteen days of life: active kormogrizein and vitamin E by 6.6% in comparison with the controls, the "low-active" kormogrizein, vitamin A and fodder biomycin by 5%. This confirms, obviously, the increased resistance of the young organism during use of the standard preparations.

The capacity of kormogrizein to stimulate the factor of natural immunity in chicks, to increase resistance of the young organism and stimulate growth and development demonstrates, apparently, the complex composition of this antibiotic preparation, which together with the antibiotic content consists of other biologically active substances (amino acids, the vitamin groups B, PP, biotin, hormones, and others).

SUMMARY

1. The antibiotic grisin (crystalline preparation) and kormogrizein (its fodder form) together with a growth-stimulating effect increase production of normal antibody-agglutinins, increase the titer blood complement and stimulate phagocytic activity of the leukocytes.

2. Grisin and active kormogrizein to a larger extent than fodder biomycin and the vitamins A and E effect the factors of natural immunity in the organism of the chick.

3. The antibiotic kormogrizein and vitamin E increase the survival rate of chicks in the first days of life with greater intensity than fodder biomycin and vitamin A.

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3 hours from the moment of injection of alpha-tocA. The effect was discounted in the alternate form. The criterion for analgesia was the absence of painful reactions (rat squeaks) in response to an electrical stimulation equal to double the threshold. Average effective doses of an analgesic and its combinations with alpha-tocA were compared.

The effect of alpha-tocA on the acute toxicity of narcotics and analgesics was determined in groups of 6 mice at 22°. Doses (concentrations) which caused death in 50% of the animals before and after the injection of alpha-tocA were compared.

Results of the study

In dosages of 0.5-1 g/kg of alpha-tocA towards the end of the third hour from the moment of injection reduced the spontaneous moving activity by 50-80-85%. In all of the animals there was a significant weakening of the orientation reflex. In doses of 0.5 g/kg, alpha-tocA caused a 60% survival rate in the base electrolyte of intraperitoneally-injected phenamine in a dose of 14 mg/kg (LD_{50}). Dosages of alpha-tocA of 1 and 2 g/kg did not change the group toxicity of phenamine.

Toward the end of the third hour alpha-tocA even in a dosage of 0.25 g/kg lessened the capacity of the central nervous system for the summation of impulses. In this same dosage it showed a weak analgesic activity. In dosages of 0.25-1 g/kg alpha-tocA moderately strengthened the activity of analgesics. The effect of morphine was most greatly strengthened. In dosages of 1 g/kg, alpha-tocA significantly strengthened the efficacy of ethyl alcohol, hexobarbital, fluorotan, and to a lesser extent, diethyl ether and Trapanal. Strengthening the effect of analgesics and narcotics on the central nervous system, alpha-tocA does not increase their toxicity.

Conclusions

1. In dosages of 0.5-1 g/kg d,l-alpha-tocopheryl acetate (alpha-tocA) decreases spontaneous moving activity in mice.

2. In dosages of 0.5 g/kg alpha-tocA decreases the mortality rate of mice from phenamine (14 mg/kg). Doses of alpha-tocA of 1 and 2 g/kg meanwhile were ineffective.

3. In dosages of 1 g/kg alpha-tocA distinctly strengthens the effect of the analgesics and narcotics on the central nervous system, meanwhile not raising their toxicity.

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Neuropharmacology of alpha-tocopherol

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Among the antioxidants alpha-tocopherol occupies a special place, taking a part in the synthesis of CoQ and CoA (Green and others, Yang and Olson), in the metabolism of serotonin (Passeri), and the developing synergism in reactions of magnesium (Selisko).

We studied the effects of alpha-tocopherol on several functions of the central nervous system.

Methods

The experiments were done with an oily solution of d,l-alpha-tocopheryl acetate (alpha-tocA) from the firm "Spofa." The preparation was tested in dosages from 0.25 to 1 g/kg. The condition of spontaneous moving activity before and after subcutaneous injections of various dosages of alpha-tocA in rats and mice was studied. In mice it produced also a collective determination of the effect of alpha-tocA on the spontaneous locomotion with the help of single-passage mazes, automatically registering the number of runs of each mouse inside the individual corridor.

Test of group toxicity of phenamine. Experiments took place in groups of 10 female mice weighing 18-24 g. The mice were placed in cages measuring 20 x 30 x 10 cm for 30 hours at 20°. Phenamine was injected intraperitoneally and determined LD_{99}. Next, the given dose (14 mg/kg) was injected by the end of the third hour of alpha-tocA influence.

The capacity of the central nervous system for the summation of nerve impulses was determined by the method of V. V. Zakusov on the basis of intramuscularly injected alpha-tocA.

The effect of alpha-tocA on the activity of narcotics was investigated using female mice weighing 18-24 g. Non-inhaled narcotics (hexobarbital, Trapanal, ethyl alcohol) were injected intravenously within 2 hours after the subcutaneous injection of alpha-tocA. The ED_{60} of narcotics was compared (test of supplementary conditions) with its combinations with alpha-tocA. For clarification of the activity of inhaled narcotics, a modified method of Jobgen and Horch was used, based on the alternative form of the registration effect (test of supplementary condition) in a group of 6 mice, placed for 10 minutes in an atmosphere saturated with narcotic smoke. Effective concentration of the matter was expressed in terms of percentages. Concentrations were compared, in which diethyl ether and Fluotan separated cause narcosis in 50% of the animals before and after injection, and in combination with alpha-tocA cause the desired effect in 50% of the cases.

The effect of alpha-tocA on the activity of analgesics was studied by the method of electrical stimulation of the tail skin of rats. Analgesics (morphine, Promedol, Phenadon) were injected, calculating to combine at the time of peak effect of each of them within

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Concerning the anti-spasmodic action of alpha-tocopherol

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In the literature there are works about the increase of tolerance to strychnine (0.1-1 mg) during the chronic injection of alpha-tocopherol in pigeons (Oggioni), about some anti-Corrazole activity of this substance, established in mice (Minsky and Giarman) for dosages on the order of 0.25 g/kg. Tocopherol decreases the toxicity of isoniazide (Prescott and coauthors).

In the present work, the anti-spasmodic activity of alpha-tocopherol was studied in greater detail.

Methods

The experiments were done with an oily soultion d,l-alpha-tocopheryl acetate (alpha-tocA) from the firm "Spofa." The preparation was injected subcutaneously. Range of the experimental dose was from .25-2 g/kg. The test subjects were white female mice weighing 18-26 g.

Antagonism to hydrazide. For clarification of the effect of alpha-tocA on the spasmodic activity of hydrazide thiosemicarbazide (30 mg/kg), semicarbazide (250 mg/kg), and Tubazide (200 mg/kg) injected subcutaneously at two hour intervals after the activity of alpha-tocA (Knoll and coauthors). They observed the "hydrazidic" spasm after diffusion and registered the length of the latent period, the number of single and repeated spasmodic attacks, and the mortality of the experimental animals.

Test of intravenous titration of Corrazole. (Orlov and co-authors; K.S. Rayevskiy). A 1% solution of Corrazole was injected at the steady rate of 0.005 ml/sec in the tail vein of the mice. The quantity of Corrazole was determined (in terms of column), necessary for the generation of a single spasmodic occurrence (shuddering, nodding, tonic extension of the posterior extremities) of intact mice and animals receiving alpha-tocA from 2 hours to the execution of the test.

Test of maximal electrical shock. The experiments were done with mice weighing 24-26 g, which from 2 hours to the execution of the test were injected subcutaneously with alpha-tocA. With the help of corneal electrodes across the head of the mice, alternating current was conducted (with a frequency of 50 periods/sec and a strength of 50 ma, continuing stimulation for 0.2 sec). The criterion for anti-spasmodic activity was the prevention of the tonic-extensor phase of the attack, originating in the response to the transmission of a current of supra-maximal strength (Toman and coauthors).

Results of the study

Alpha-tocA in dosages on the order of 0.5 g/kg and more prolonged the latent period of the spasm, increasing the number of repeated spasmodic attacks, and did not change the lethality of the

animals receiving semicarbazide and Tubazide. The same dosages of alpha-tocopherol acetate in combination with thiosemicarbazide caused an essential prolongation of the latent period of convulsion, cutting spasmodic attacks, 28-40% survival rate of mice after an injection of an absolute fatal dose of hydrazide. With a base electroshock of alpha-tocA in dosages of 1.8 g/kg even a lengthening of the latent period of the spasm did not take place (Table I).

Investigation of the antagonism of alpha-tocA to Corazole was apparently a clear protective effect from a dosage of 1g/kg alpha-tocA tolerated an injection of 0.2 ml Corazole in comparison with 0.14 ml in the control (higher spasmodic threshold in 1.42 times). The substance studied in dosages of 0.5 g/kg possessed distinct protective characteristics (prevention of the tonic extensoric phase) as regards the gross depolrized effect of the maximal electric shock.

However, with an increase in dosage the protective effect decreased (Table II).

Discussion of the results

In our previous work (F. N. Uskov) the sedative effect of alpha-tocA was established, the strength of which varied in relation to the available dosage. In the present study the cleaving effect of alpha-tocA is recorded during the use of dosages on the order of 0. , 1, and 2 g/kg.

Such variations in the influence of the specified groups of dosages demanded clarification.

French investigators calculated that several antioxidants operate as the means of alleviating nutrient deficiencies CoA, CoQ, NAD (coenzyme A, ubiquinone, nicotinamide-adenine-dinucleotide) in reactions (laborit and coauthors, 1960). It is important to note that alpha-tocopherol is closely related to the indicated higher factors (Yang and Olson; Green and coauthors; Barbari). Determination of the effect of alpha-tocA on the process of cellular metabolism favors assuming the biochemical heterogeneity of the investigated effects of this substance in the central nervous system.

The speed of the conversion reaction of alpha-keto-glutamic acid in succinic acid in the Krebs cycle is constant and does not depend on the speed of the course of the previous stages of the cycle. This is explained by the occurrence of the shunting mechanism of alpha-keto-glutamic acid- glutamic acid- GABA. Alpha-tocopherol causes acceleration of the course of the citric acid cycle owing to: 1) facilitated electron transfer in the standard chain of respiratory enzymes by means of direct sharing between the b and c cytochromes (Mason and Lehman) or transmitted across CoQ (Green and coauthors , 2) saturation of the cycle with malate in the result of the facilitation of the course of the reaction "pyruvate-malate," causing acceptance of hydrogen with NADP, Mg (nicotinamide-adenine-dinucleotide-phosphate) recovered (Laborit, 1961). By such a method the circumstances for the triggering of the shunting mechanism can be determined. If we also take into account the circumstance that alpha-tocopherol possibly accelerates the course of its own reaction with alpha-keto-glutamic acid- succinic acid, that indirectly confirms the important increased percentage of glutamic acid in the tissues of the brain of vitamin-E deficient animals (Faith and Nelson, 1957), that clearly, under these conditions the shunting mechanism becomes still more effective (see figure).

Everything said helps to evaluate the results we received. Thiosemicarbazide, according to Knoll and his coauthors, blocks selectively pyridoxal-phosphate co-enzyme decarboxylase L-glutamic acid.

1. Антагонизм α-токоферола с судорожными гидразидами

2. Соединение	3. Доза (в мг/кг)	4. Доза α-токоферола (в г/кг)																	
		0			0,25			0,5			0,75			1			2		
		5. л	6. с	7. п	5. л	6. с	7. п	5. л	6. с	7. п	5. л	6. с	7. п	5. л	6. с	7. п	5. л	6. с	7. п
8. Тиосемикарбазид	30	70	100	100	90	80	73,3	130	80	60	90	93	80	72	100	100	71	100	100
9. Семикарбазид . . .	250	68	80	73,3	75	73,3	60	80	93,3	80	70	80	60	70	80	73,3	67	80	73,3
10. Тубазид	200	60	66,6	40	65	73,3	40	80	80	46,6	65	73,3	40	58	66,6	40	60	73,3	46,6

11. Примечание. Каждая доза α-токоферола изучена на 15 мышах; л — средняя величина латентного периода судорог (в мин.), с — число животных с судорогами (в %), п — число погибших животных (в %).

Table I.

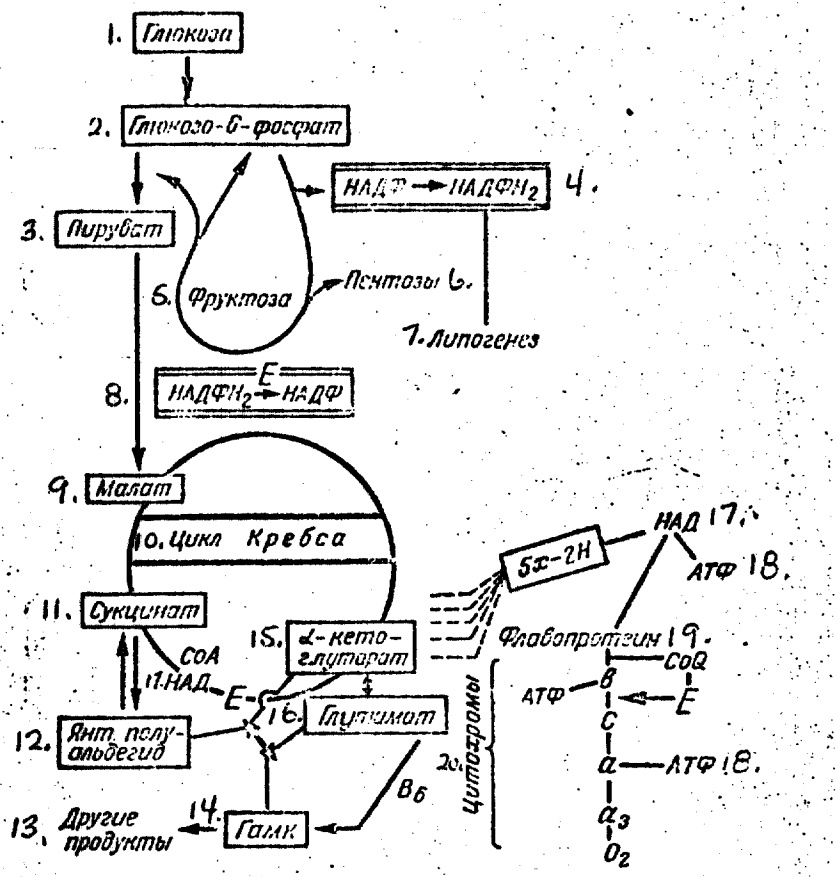
1) Antagonism of alpha-tocopherol to spasmodic hydrazide; 2) compound; 3) dosage (in mg/kg); 4) dosage of alpha-tocopherol (in g/kg); 5) l; 6) s; 7) p; 8) thiosemicarbazide; 9) semicarbazide; 10) Tubazide; 11) Note. Each dosage of alpha-tocopherol was administered to 15 mice; l- average length of the latent period of the spasm (in minutes); s- number of animals with spasms (in %); p- number of dead animals (in %).

1. Предупреждение α-токоферолом тонико-экстензорной фазы судорог (тест максимального электрошока). Каждая доза изучена на 10 мышах

2. Характер судорог	3. Доза α-токоферола (в г/кг)					
	0	0,25	0,5	0,75	1	2
4. Тонико-экстензорная фаза (в %)	100	70	50	60	80	90
5. Клонус (в %)	100	100	100	100	100	100

Table II.

1) Prevention by alpha-tocopherol of the tonic-extensoric phase of the spasm (test of maximal electrical shock). 2) Each dosage administered to 10 mice; 2) type of spasm; 3) dosage of alpha-tocopherol (in g/kg); 4) tonic extensoric phase (in %); 5) clonus (in %).



21. Упрощенная схема превращения глюкозы (по Laborit).
E — основные уровни участия α-токоферола в процессах обмена.

Figure.

1- Glucose; 2- glucose-6-phosphate; 3- pyruvate; 4- NADP— $NADPH_2$; 5- fructose; 6- pentoses; 7- lipogenesis; 8- $NADPH_2$— NADP; 9- malate; 10- Krebs cycle; 11- succinate; 12- succinic aldehyde; 13- other products; 14- GAMC; 15- alpha-ketoglutarate; 16- glutamate; 17- NAD; 18- ATP; 19- flavoprotein; 20- cytochromes; 21- simplified system of conversion of glucose (according to Laborit). E- basic levels of participation of alpha-tocopherol in the processes of metabolism.

causing a [illegible] deficit of GAMC in the brain tissues, spasms and death of animals (dose of 5[illegible] mg/kg). First the injected alpha-tocopherol forms the GA Cic base electrolyte by the method described above, sharply extending the latent period of convulsion causing a mortality rate of 28-40% among the experimental animals (dosage of alpha-tocopherol on the order of 0.5 g/kg). The spectrum of the effect of semicarbazide and Tubazide is apparently wider and not organized in a unit of specified decarboxylases. That is why in combination with them antagonism to GA Cic components is observed no sooner than their effects (somewhat lengthened latent period of the spasm, and increase of frequency in the spasmodic attack without an effect on the mortality of the animals).

In that the GA-Cic mechanism has an essential significance in the protective effect of alpha-tocopherol in these same doses, we will also explain the data from the maximal electrical shock experiment (gross depolymerizing agent). Besides this it is known that GABC plays a large role in the processes of hyperpolarization (Laborit, 1961). Reduction in the protective effect is established during the application of a dose larger than 0.5 g/kg. Dosages on the order of 2 g/kg appeared to be ineffective, apparently, in the results of the molecular plotting of the GA-Cic base electrolyte on account of the transfer of the surplus GAMC, for example the succinic semialdehyde in succinic acid (see figure). Saturation of the Krebs cycle by succinate causes it to work still more intensively, leading to the earliest depletion of reserves of NADPH$_2$. Applicable premises are set up for the combustion of glucose-6-phosphate into a pentose on account of the inhibition of exergonic method. Thus, it is an entirely possible hypothesis that in effect a massive dose of alpha-tocopherol plays an important role in the "pentosis" mechanism.

An indirect confirmation is developed in the biochemical investigations of Gray (1959, 1960), where the increasing tissue concentration of phospholipids and nucleoproteins under the effect of massive doses of alpha-tocA was demonstrated. Besides this it is known that these synthetic processes are connected with the functional course of the pentose. Furthermore there is still one indirect confirmation of our hypotheses. Chlorpromazine in experiments with spasmodic hydrazides appeared to be absolutely ineffective (Knoll and coauthors). On the other hand, it is apparent that chlorpromazine appears as an activator of the course of the pentose (Forrest and coauthors 1958, cited in Laborit, 1961). A clear reduction of excitability of the central nervous system in experiments with Corazole also speak of the pentosis mechanism influence of massive doses of alpha-tocopherol.

We investigated in previous works the sedative effect of alpha-tocopherol, which appeared diverse in strength of dependence on applied dosages (F. N. Uskov). In the present work we have the bases for a hypothesis of the non-uniform mechanism of reaction of the central nervous system for dosages on the order of 0.5 and 2 g/kg.

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SYNERGISM OF d- AND l-α-TOCOPHEROL*) DURING ABSORPTION

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In a preceding communication (Weber et al., 1963), various biological aspects of d-α- and l-α-tocopherol were compared. As far as their distribution in various tissues and their metabolic degradation is concerned, no marked differences between the two compounds could be found. However, some differences were observed in the absorption of the compounds inasmuch as l-α-tocopherol was absorbed more rapidly than the d-form. It must, therefore, be considered that this behaviour may affect the absorption of d-α- and l-α-tocopherol when administered as a mixture, all the more as it is known that the intestinal absorption is a limiting factor for the biological availability of α-tocopherol. To study this problem, it was necessary to distinguish between the single components of such a mixture by analytical procedures. For that reason, a mixture of differently labeled α-tocopherols, i.e. of carbon-labeled d-α-tocopherol and ^{3}H-labeled l-α-tocopherol, was used for these studies.

2 mg of the mixture (1 : 1) of (5-methyl-^{14}C)-d-α-tocopheryl acetate (specific activity = 1.6 μC/mg) and (1',2'-^{3}H)-l-α-tocopheryl acetate (specific activity = 99.7 μC/mg) were administered to rats as described previously (Weber et al., 1963) and were compared with 2 mg of ^{3}H-labeled d-α- and 2 mg of l-α-tocopheryl acetate. To determine the radioactivity, aliquots of the tissue homogenates and of the blood samples were combusted (Kalberer and Rutschmann, 1961) and the two isotopes were counted in the same sample according to the discriminator-ratio method of Okita et al. (1957) using a Packard Tri-Carb scintillation counter. To render a direct comparison possible, the radioactivity measured in the various tissues was converted into weight units for both ^{14}C-d-α- and ^{3}H-l-α-tocopherol.

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*) The d-α- and l-α-tocopherol preparations used correspond to (2R,4'R,8'R)-α- and (2S,4'RS,8'RS)-α-tocopherol, resp. (Mayer et al., 1963).

From the results shown in Fig. 1 and 2, it can be seen that the absorption of the mixture of d- and l-α-tocopherol was considerably higher (about 55 % on the average) than it was to be expected from the data obtained after administration of the single compounds. The increase of the absorption of the mixture concerns both d-α- and l-α-tocopherol in about the same ratio.

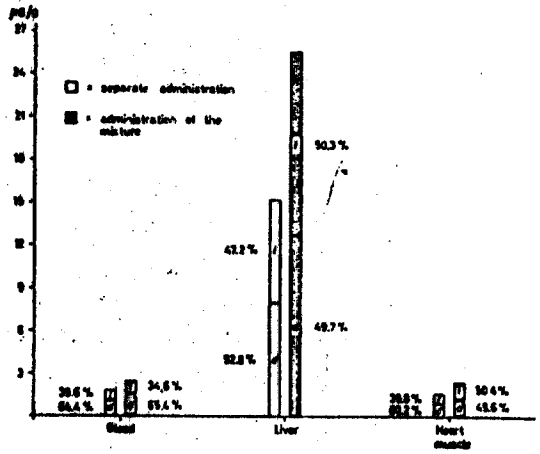

Fig.1. The α-tocopherol content of blood, liver and heart muscle of rats, 2 hours after the oral administration of 2 mg of a mixture of ^{14}C-d-α- and ^{3}H-l-α-tocopheryl acetate and after separate dosing of 2 mg of ^{3}H-d-α- or ^{3}H-l-α-tocopheryl acetate. In order to render a direct comparison possible, the values obtained for d-α- and l-α-tocopherol administered separately were halved and put together in one column. Average of two animals.

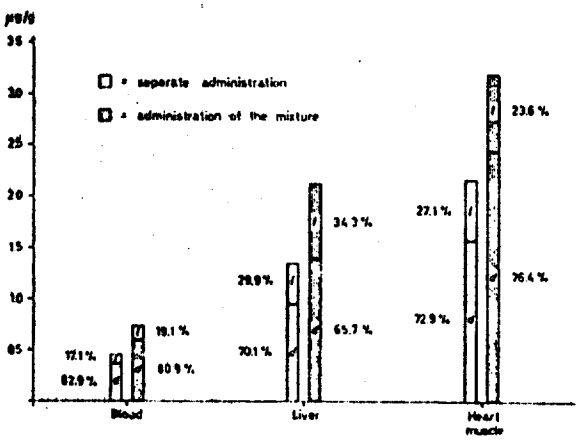

Fig.2. Comparison of the α-tocopherol content of blood, liver and heart muscle of rats, 96 hours after the oral administration of labeled d-α- and l-α-tocopheryl acetate, mixed or separately, as described under Fig.1.

In virtue of these results, it must be considered that the calculation of the biopotency of dl-α-tocopherol from that of the single components may not be justified without taking into account the different behaviour of the mixture and of the single compounds during absorption.

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STUDIES ON THE ABSORPTION, DISTRIBUTION AND METABOLISM OF l-α-TOCOPHEROL*) IN THE RAT

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From previous extensive studies comparing d-α-tocopherol with dl-α-tocopherol by various biological assay methods, it was concluded that the biopotency of l-α-tocopherol is lower than that of the d-form. Based on these results, a conversion factor of 1.22 to 1.36 for the biopotency of d-α-tocopherol in relation to dl-α-tocopherol was established (Nutr.Revs. 19, 146 (1961)). The availability of pure l-α-tocopherol obtained by chemical synthesis (Mayer et al., 1963) facilitated the examination whether besides quantitative also qualitative differences exist between the d- and l-form of α-tocopherol.

Simon et al. (1956) have found that, after administration of d-α- and dl-α-tocopherol, the glucuronide of the γ-lactone of 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethyl-1,4-benzoquinone was excreted in the urine of human beings and rabbits. The same metabolite could now be detected in the urine of rats dosed with labeled d-α- or l-α-tocopherol, indicating that no differences exist in the metabolism of these two forms of α-tocopherol. Up to now, however, it was not possible to decide whether an inversion of the configuration occurs during the metabolic degradation. In these experiments, 2 mg-doses of (1',2'-^{3}H)-d-α- and (1',2'-^{3}H)-l-α-tocopheryl acetate (specific activity = 101.9 μC/mg and 106.2 μC/mg, resp.) were administered orally to fasted rats (weighing about 140 to 150 g) as an emulsion in 0.5 ml of 0.9 % saline containing 2 % Tween 80. The urine was collected for a period of four days and the metabolite identified as described previously (Weber and Wiss, 1963).

The absorption of d-α- and l-α-tocopheryl acetate was studied by determining the radioactivity in various organs, half an hour, two and

*) The d-α- and l-α-tocopherol preparations used correspond to (2R,4'RS,8'RS)-α- and (2S,4'RS,8'RS)-α-tocopherol, resp., synthesized by Mayer et al. (1963).

twenty-four hours after administration. The results are shown in Fig. 1 to 3 from which it can be seen that l-α-tocopherol is absorbed better and excreted somewhat more rapidly than the d-form. The better absorption of l-α-tocopherol is confirmed by a higher excretion of the metabolite, the amount of which was found to be about three times higher after administration of l-α-tocopherol than after administration of d-α-tocopherol within a period of twenty-four hours.

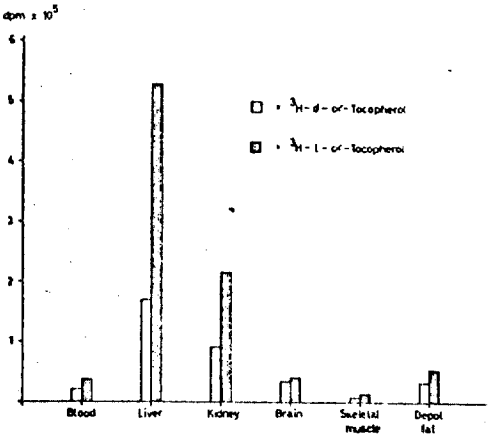

Fig.1. Distribution of the radioactivity (dpm/g fresh tissue) in blood, liver, kidney, brain, skeletal muscle (from the thigh), and depot fat (testicular fat) of rats, half an hour after the oral administration of 2 mg of ^{3}H-d-α- or ^{3}H-l-α-tocopheryl acetate. The columns represent mean values of three animals each.

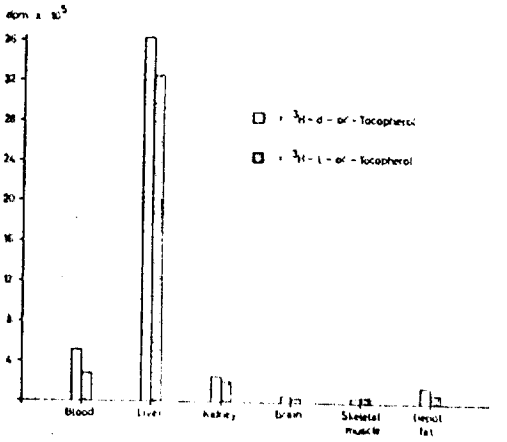

Fig.2. Distribution of the radioactivity (dpm/g fresh tissue) in various tissues of rats, 2 hours after the oral administration of 2 mg of ^{3}H-d-α- or ^{3}H-l-α-tocopheryl acetate. Mean values of two animals each.

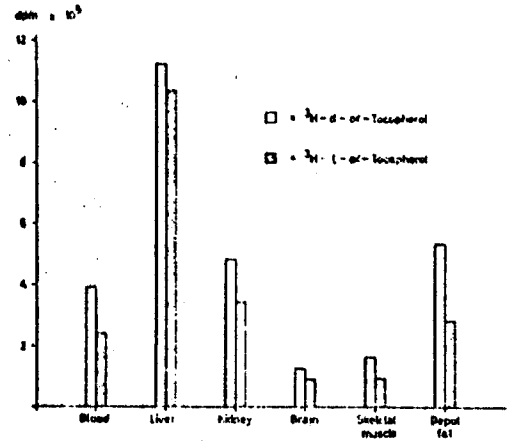

Fig.3. Distribution of the radioactivity (dpm/g fresh tissue) in various tissues of rats, 24 hours after the oral administration of 2 mg of ^{3}H-d-α- or ^{3}H-l-α-tocopheryl acetate. Mean values of two animals each.

In previous investigations (Wiss et al., 1962), it was shown that d-α-tocopherol exhibits a characteristic distribution pattern in various organs and cell particles which differs markedly from that of the unnatural antioxidant ethoxyquin*). It was, therefore, of interest to compare d-α- and l-α-tocopherol in this respect. As shown in Fig. 2, the distribution patterns of d-α- and l-α-tocopherol are very similar. The same could be found when comparing the accumulation of α-tocopherol in the adrenals. Twenty-four hours after administration, the concentration of d-α-tocopherol in the adrenals was 37 times higher than in the blood (on a weight basis), whereas the corresponding factor for l-α-tocopherol was found to be 32.

By separating the cell particles of liver homogenate, it was previously demonstrated that d-α- and dl-α-tocopherol were especially enriched in the microsomes (Cowlishaw et al., 1957; Draper and Alaupovic, 1959; Weber et al., 1962). Comparative studies with ^{3}H-labeled d-α- and l-α-tocopherol revealed that the same applies to l-α-tocopherol.

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LUPUS ERYTHEMATOSUS

Treatment by Combined Use of Massive Amounts of Calcium Pantothenate or Panthénol* with Synthetic Vitamin E

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CINCINNATI

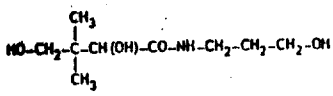
THIS REPORT deals with the results obtained in the treatment of certain phases of lupus erythematosus by the administration of combinations of various forms of vitamin E with either panthenol* (the alcohol of pantothenic acid), racemic (*dl-*) calcium pantothenate or dextrorotatory (*d-*) calcium pantothenate. Patients who had chronic discoid, disseminate discoid, or subacute disseminate lupus erythematosus were studied. Patients with acute disseminate lupus erythematosus were excluded.

Racemic calcium pantothenate has been used alone, in doses of 200 to 400 mg. daily for short periods, with benefit in the treatment of chronic discoid, disseminate discoid, and subacute disseminate lupus erythematosus.[1] Panthenol* alone, in doses varying from 0.5 to 6.0 gm. daily for periods varying from two weeks to three months has been used with improvement in all types of lupus erythematosus.[2] Vitamin E alone has been used with improvement in the treatment of lupus erythematosus.[3]

There is no report in the literature of the use of the combination of the drugs employed in this study in the treatment of patients with lupus erythematosus. In addition we could find no record of the use of these drugs in the heavy dosage or for the long intervals here employed.

DRUGS EMPLOYED

Panthenol,®[4] the alcohol corresponding to pantothenic acid, which is chemically *d*-alpha, gamma-dihydroxy-N-(3-hydroxypropyl)beta, beta-dimethylbutyramide, has the following structural formula:



Read before the Seventy-First Annual Meeting of the American Dermatological Association, Hot Springs, Va., May 25, 1951.

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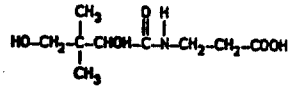
4. The panthenol* used in this study was supplied by Hoffman-LaRoche, Inc.

It is considerably more stable than pantothenic acid in the pH range 2 to 5.[6] It is the most active analogue of pantothenic acid hitherto described which displays vitamin activity. The medial lethal dose of panthenol* for mice upon oral administration was found to be approximately 15 gm. per kilogram. Panthenol,* given orally, is converted to pantothenic acid, as judged by the urinary excretion of the latter compound. It was administered in 0.5-gm. capsules. Calcium pantothenate was used as the dextrorotatory salt[6] and as the combination of the dextrorotatory and the levorotatory salts.[7] The formula of pantothenic acid, which is formed from beta, beta-dimethyl-alpha-hydroxybutyrolactone condensed with beta-alanine, is as follows:



The calcium salt is simply this formula with the last hydroxyl radical replaced by calcium. The dextrorotatory calcium salt of pantothenic acid, $(C_9H_{16}NO_5)_2Ca$, is the biologically active form. The levorotatory form is biologically inactive. The biological activity of the *dl*-calcium pantothenate used here was 49% that of the dextrorotatory form, as shown by the microbiological test for pantothenic acid.[8] The medial lethal dose of the synthetic dextrorotatory calcium pantothenate for mice, when given orally, was found to be approximately 10 gm. per kilogram.[9]

Pantothenic acid is essential for the nutrition of many species of animals. It is not known whether it is essential for human nutrition, although this is assumed.[10] The pantothenic acid content of normal human blood varies from 3 to 19.4 γ per 100 cc. In persons suffering from pellagra, beriberi, and riboflavin deficiency, blood levels of pantothenic acid are from 23 to 50% lower than in normal persons.[11] The requirements are increased in experimental hyperthyroidism.[12] There is some evidence that pantothenic acid may be concerned with fat and carbohydrate metabolism, particularly the rate of pyruvate oxidation in the liver.[13]

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Jürgens and Pfaltz [14] demonstrated that in rats pantothenic acid is an essential factor for the normal function of epithelial tissue. Among the effects attributed to pantothenic acid deficiency are nutritional achromotrichia,[15] dermatitis,[16] gastritis, enteritis, and ulcers of the intestinal tract,[17] hemorrhagic necrosis of the adrenal glands,[18] hemorrhagic degeneration of the testes [18] and kidneys,[17a] nerve degeneration,[19] and inhibition of growth.[20] In humans pantothenic acid seems to be combined with riboflavin [21] and inositol metabolism.[22] Folic acid and biotin may be essential for the utilization of pantothenic acid.[23] Pantothenic acid deficiency in experimental animals reduces considerably the phagocytic power of the blood.[24] Pantothenic acid analogues have an antibacterial action.[25]

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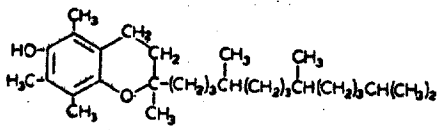
Spies and his associates [10b] administered varying amounts of sodium and calcium pantothenate to 15 subjects, and observed that 100 mg. of either salt may be injected intravenously without causing any significant change in blood pressure, pulse, temperature, or respiration, or any other noticeable reaction.

Studies by Unna and Greslin [9] on the toxicity of synthetic dextrorotatory calcium pantothenate caused the authors to conclude that calcium pantothenate, like the other members of the vitamin B complex, is a substance of extremely low toxicity. Excessive large doses produce death by respiratory failure in mice and rats, but prolonged feeding of sublethal doses fails to produce toxic manifestations. These facts indicate that an excess of pantothenic acid is either rapidly destroyed or excreted. In view of the foregoing investigations and many others, it will be seen that there is no basis for stating a proper dose of pantothenic acid or its salts.

Vitamin E was used as the natural mixed tocopherols and as the synthetic *dl*-alpha-tocopherol acetate (ephynal acetate®).[26] The structural formula of the alpha-tocopherol is



Beta-, gamma- and delta-tocopherols are isomers, possessing one less methyl group than alpha-tocopherol in the aromatic nucleus. Alpha-tocopherol has proved to be most effective, biologically. The dextrorotatory form of the tocopherols and the tocopherol acetate esters are much more active biologically than the racemic forms. Dextrorotatory alpha-tocopherol acetate is the most active form now known. Ephynal acetate® is the acetate ester of racemic alpha-tocopherol.

One of the striking chemical properties of vitamin E is its activity as an antioxidant.[27] It has a protective or "sparing" action in preventing the oxidation of vitamin A and carotene.[28] It also has an antidystrophic action on muscle tissue.[29]

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26. The ephynal acetate® used in this study was supplied by Hoffmann-LaRoche, Inc.

27. Vitamin E as a Physiological Antioxidant, Nutrition Rev. **3**:17, 1945.

28. Krakower, C., and Axtmayer, J. H.: Effect of Alpha-Tocopherol on Lesions of Skeletal Muscles in Rats on Vitamin A Deficient Diets, Proc. Soc. Exper. Biol. & Med. **45**:583, 1940. Moore, T., and Davis, A. W.: Interaction of Vitamins A and E, Nature **147**:794, 1941. Shantz, E. M.; Cawley, J. D., and Embree, N. D.: Anhydro ("Cyclized") Vitamin A, J. Am. Chem. Soc. **65**:901, 1943. Hickman, K. C. D.; Kaley, M. W., and Harris, P. L.: Convitamin Studies: II. The Sparing Action of Natural Tocopherol Concentrates on Vitamin A; III. The Sparing Equivalence of the Tocopherols and Mode of Action, J. Biol. Chem. **152**:303, 313, and 321, 1944.

29. (*a*) Pappenheimer, A. M.: Certain Nutritional Disorders of Laboratory Animals Due to Vitamin E Deficiency, J. Mt. Sinai Hosp. **7**:65, 1940. (*b*) Martin, A. J. P., and Moore, T.: Some Effects of Prolonged Vitamin E Deficiency in the Rat, J. Hyg. **39**:643, 1939. (*c*) Goettsch, M., and Pappenheimer, A. M.: Nutritional Muscular Dystrophy in Guinea Pig and Rabbit, J. Exper. Med. **54**:145, 1931. (*d*) Evans, H. M., and Burr, G. O.: Development of Paralysis in the Sucking Young of Mothers Deprived of Vitamin E, J. Biol. Chem. **76**:273, 1928. (*e*) Moore, T., and Wang, Y. L.: The Fluorescence of the Tissues in Avitaminosis E, Proc. Biochem. Soc., Biochem. J., 1943, vol. 37, p. 1. (*f*) Kaunitz, H., and Pappenheimer, A. M.: Oxygen Consumption in Vitamin E [illegible] Am J Physiol 138:322, 1943

Vitamin E has been reported to have some action on both male and female gonads,[30] on other endocrine glands,[31] on nervous tissues,[32] on muscles,[33] on vessels,[34] and on growth.[35] The recommended dosage of vitamin E varies from 10 mg. to 600 mg. daily for the multiple purposes for which it has been used. There is some slight and indirect evidence that vitamin E can be toxic.[36] Vitamin B increases tolerance for vitamin E.[37] Rancid fats destroy vitamin E.[38]

30. Martin and Moore.[29b] Evans and Burr.[29d] Kaunitz and Pappenheimer.[29f] Evans, H. M.; Burr, G. O., and Althausen, T. L.: The Antisterility Vitamin Fat Soluble E, Memoirs of the University of California, Vol. 8, Berkeley, University of California Press, 1927. Pappenheimer, A. M., and Schogoleff, C.: The Testis in Vitamin E-Deficient Guinea Pigs, Am. J. Path. **20**:239, 1944. Barrie, M. M. O.: The Effect of Vitamin E Deficiency in the Rat: I. Duration of Gestation; II. Lactation; III. Fertility in the Female, Biochem. J. **32**:1467, 1474, and 2134, 1938; V. Uterine Changes in Chronic Deficiency, ibid. **37**:523, 1943. Bacharach, A. L., and Allchorne, E.: Investigations into the Method of Estimating Vitamin E: Further Observations on Vitamin E Deficiency and Implantation, ibid. **32**:ii, and 1298, 1938. Mason, K. E.: Differences in Testis Injury and Repair After Vitamin A Deficiency and Inanition, Am. J. Anat. **52**:153, 1933.

31. (*a*) Drummond, J. C.; Noble, R. L., and Wright, M. D.: Studies in the Relationship of Vitamin E (Tocopherols) to the Endocrine System, J. Endocrinol. **1**:275, 1939. (*b*) Barrie, M. M. O.: The Relation of Vitamin E to the Anterior Lobe of the Pituitary Gland, Lancet, **2**:251, 1937. (*c*) Nelson, W. O.: Studies on Anterior Hypophysis: The Anterior Hypophysis in Vitamin E-Deficient Rats, Anat. Rec. **56**:241, 1933. (*d*) Kepinov, L.: Les vitamines A et E, agents excitants de la sécrétion de l'hormone glycogénotrope du lobe antérieur de l'hypophyse, Compt. rend. Acad. sc. **210**:188, 1940. (*e*) Tonutti, Von E.: Über die Nebennierenrinde bei Vitamin E-freier Ernährung, Ztschr. Vitaminforsch. **13**:1, 1943.

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33. Burr, G. O.; Brown, W. R., and Moseley, R. L.: Paralysis in Old Age in Rats on a Diet Deficient in Vitamin E, Proc. Soc. Exper. Biol. & Med. **36**:780, 1937. Mackenzie, C. G.: Cure of Repeated Attacks of Nutritional Muscular Dystrophy in the Rabbit by Alpha-Tocopherol, ibid. **49**:313, 1942. Gullickson, T. W., and Calverley, C. E.: Cardiac Failure in Cattle on Vitamin E-Free Rations as Revealed by Electrocardiograms, Science, **104**:312, Oct. 4, 1946. Martin, G. J., and Faust, F. B.: The Heart in Avitaminosis E, Exper. Med. & Surg. **5**:405, 1947.

34. Shute, E. V.; Vogelsang, A. B.; Skelton, F. R., and Shute, W. E.: The Influence of Vitamin E on Vascular Disease, Surg., Gynec. & Obst. **86**:1, 1948.

35. Barrie.[31b] Nelson, W. O., and others: Growth Stimulating Activity of Alpha-Tocopherol, Proc. Soc. Exper. Biol. & Med. **45**:157, 1940. Emerson, G. A., and Evans, H. M.: Successive Generations of Vitamin E-Low Rats, ibid. **45**:149, 1940.

36. Weissberger, L. H., and Harris, P. L.: Effect of Tocopherols on Phosphorus Metabolism, J. Biol. Chem. **151**:543, 1943. Shute, E.: A Reply to Recent Criticisms of the Theory of a Relationship Between Vitamin E and the Oestrogens, J. Endocrinol. **2**:173, 1940. Bicknell, F.: Vitamin E in the Treatment of Neurological Conditions, in The Vitamins in Medicine, ed. 2, New York, Grune & Stratton, Inc., 1946, p. 767.

37. Bicknell, F.: Amyotrophic Lateral Sclerosis and Progressive Muscular Atrophy, in The Vitamins in Medicine, ed. 2, New York, Grune & Stratton, Inc., 1946, p. 768.

38. Mattill, H. A.: Muscular Dystrophy in Rabbits and the Auto-Oxidation of Fats, in Proceedings of the Seventh Annual Meeting of the American Institute of Nutrition

TREATMENT PROGRAM

When this study was begun, patients with lupus erythematosus were treated with *d*-calcium pantothenate (100-mg. capsules) alone. The original dosage varied from 0.5 gm. to 2.0 gm. daily. Some improvement was noted. Vitamin E, in the form of natural tocopherols (100-mg. capsules), was then added to the therapeutic program. The original dosage was 300 mg. daily. The dosage of *d*-calcium pantothenate was increased to 3 gm. daily, and the dosage of the natural tocopherols to 400 mg. daily. More rapid improvement was noted in patients treated at once with this higher dosage schedule than in those who started with the lower dosages. Patients who had improved for a time and then remained stationary on the lower dosage schedule began to progress favorably when put on the higher dosage schedule.

Panthenol® was then obtained for use of patients who had lupus erythematosus. At first, this was supplied in 150-mg. capsules, but later in 0.5-gm. capsules. In the early use of this agent, the daily dose was started at 2.0 gm. and slowly increased every two or three weeks to 8 to 12 gm. In later patients, the starting daily dosage was 4.0 gm., and the dosage was increased rapidly over two or three weeks to the 10-to-12-gm. level. *dl*-Calcium pantothenate became available in 0.5-gm. capsules, and we proceeded to use it in the same manner as panthenol.® Ephynal acetate® was then substituted for the natural tocopherols in the treatment schedule. This drug was supplied in 100-mg. capsules. Treatment was started with 400 to 600 mg. of ephynal acetate® daily, and the dosage was rapidly increased to 800 to 1,000 mg. daily.

Many of the patients treated with panthenol® or *dl*-calcium pantothenate with ephynal acetate® had already been taking *d*-calcium pantothenate and natural tocopherols. In order to maintain an uninterrupted treatment program, patients who started treatment with panthenol® were given *d*-calcium pantothenate or *dl*-calcium pantothenate when supplies of panthenol® were short. Conversely patients who began with *dl*-calcium pantothenate or *d*-calcium pantothenate were given panthenol® when supplies of these "starting" drugs were short. Patients were asked to resume natural tocopherols when supplies of ephynal acetate® were short. Consequently, these patients were treated with some source of pantothenic acid and some form of vitamin E for long intervals, with few interruptions.

CLINICAL MATERIAL STUDIED

Forty-two patients[39] who had lupus erythematosus were treated with the above drugs in various doses. Of these patients, 24 had chronic discoid, 11 had disseminate discoid, and 7 had subacute disseminate lupus erythematosus. Ten patients either disappeared from observation before adequate treatment could be

Nutrition, 1940, Vol. 19, p. 13, Mattill, H. A.: Vitamin E, J. A. M. A. **110**:1831 (May 28) 1938. Cummings, M. J., and Mattill, H. A.: The Auto-Oxidation of Fats with Reference to Their Destructive Effect on Vitamin E, J. Nutrition **3**:421, 1931. Mackenzie, C. G.: Levine, M. D., Rabbit by Alpha Tocopherol in the Absence of a Water-Soluble Factor, ibid. **20**:399, 1940. and McCollum, E. V.: The Prevention and Cure of Nutritional Muscular Dystrophy in the Mackenzie, C. G., and others: Uncomplicated Vitamin E Deficiency in the Rabbit and Its Relation to the Toxicity of Cod Liver Oil, ibid. **21**:225, 1941.

39. Two of these patients were treated in the department of dermatology and ... of the U. of California at Cincinnati General Hosp

given, or had so many lengthy interruptions in the program that the treatment course was considered wholly inadequate and unsatisfactory. These 10 patients are therefore not included in the following appraisal of the treatment program outlined above. Of the 32 remaining patients who are included in this report, 17 had chronic discoid, 9 had disseminate discoid, and 6 had subacute disseminate lupus erythematosus. Twenty-three of this group had had previous treatment for lupus erythematosus, including gold preparations, bismuth, liver, oxophenarsine (mapharsen®) hydrochloride and sodium *p*-aminobenzoate. Some of these had had treatment for many years. The remaining nine patients had had no previous treatment for lupus erythematosus.

Of patients with the chronic discoid type many had had the disease from 9 to 29 years. Some already had considerable amounts of disfiguring scar-

TABLE 1.—*Treatment of Patients Who Had Chronic Discoid Lupus Erythematosus*

Case No. and Initials	Sex	Duration of Disease, Yr.	Previous Treatment	Duration of This Treatment, Mo.	*d*-Calcium Pantothenate, Gm.	*dl*-Calcium Pantothenate, Gm.	Panthenol,* Gm.	Natural Tocopherols, Gm.	Ephynal Acetate,* Gm.	Per Cent of Clearing	Complications of Treatment
1, J. G.	F	3	Yes	29	901.3	100.0	1,700.5	269.5	48.3	95	None
2, T. J. G.	M	9	Yes	21	562.8	.....	3,169.5	89.4	139.4	100	G. I.*
3, E. H.	F	10	Yes	20	1,020.0	68.4	46.5	285.6	138.6	100	None
4, E. H. H.	F	9	Yes	36	431.0	.....	2,073.4	141.5	135.5	99	None
5, H. J.	F	25	Yes	24	76.8	.....	3,122.6	123.9	163.5	85	None
6, L. L.	M	4	Yes	10	.....	.....	1,777.0	.....	165.2	85	None
7, R. I. R.	M	11	No	36	477.2	108.0	20.0	.....	58.0	75	G. I.*
8, R. R.	M	29	Yes	24	374.0	200.0	2,412.3	86.6	225.0	90	None
9, L. S.	F	6	Yes	30	1,254.0	.....	1,417.0	8.8	85.2	80	None
10, M. S.	F	2	No	17	1,271.2	.....	1,225.0	30.8	146.4	90	None
11, T. S.	F	10	Yes	12	.....	72.0	873.6	.....	70.2	70	None
12, L. T.	F	8	No	22	730.6	.....	100.7	182.7	.....	100	None
13, L. J.	F	10	Yes	30	1,519.8	150.0	1,109.0	120.6	.....	95	None
Von W. 14, W. R. W.	M	1.3	No	17	731.7	.....	1,066.0	.....	79.0	80	None
15, D. Z.	F	2	No	30	1,155.8	100.0	.....	153.2	.....	100	None
16, H. W.	M	9	Yes	11	259.4	.....	30.0	75.0	.....	70	None
17, E. H. W.	F	6	Yes	16	392.8	.....	.....	59.9	.....	100	None

* Transient gastric distress from panthenol.

ring. Several had heavy, markedly infiltrated, and hyperkeratotic lesions. Five patients, who had chronic discoid lupus erythematosus when the above treatment was started had experienced in the past episodes of the disseminate discoid type. Four with the disseminate discoid type had subjective systemic symptoms consisting of fatigue, anorexia, arthralgia, and weight loss, as well as objective systemic symptoms consisting of low white blood cell count, relative and absolute lymphocytosis, reversed ratio of albumin to globulin, false-positive serologic reactions, etc. The duration of the disease in patients who had disseminate discoid lupus erythematosus (Table 2) and in patients who had subacute disseminate lupus erythematosus (Table 3) represents the total interval of the disease in these patients, and not just the duration of the attack present when this form of treatment was instituted. Some of the patients in each group had several episodes over a period of years.

The duration of treatment by the combined use of the drugs studied, as indicated in the tables, does not represent continuous treatment throughout

these patients. In many instances, a good part of the early course of treatment consisted in the use of very small doses of the drugs used. In some patients the maximum dosage schedule was employed for only the last few months of the treatment course. The shortest treatment period in the series was two months. The longest treatment period in the series was three years.

In patients in whom treatment was interrupted the disease ceased to progress favorably and, if treatment was stopped for too long, relapsed in the old sites, and in some instances new lesions developed. New lesions developed in five patients while they were under treatment with the various combinations of vitamin E and

TABLE 2.—*Treatment of Patients Who Had Disseminate Discoid Lupus Erythematosus*

Case No. and Initials	Sex	Duration of Disease, Yr.	Previous Treatment	Duration of This Treatment, Mo.	*d*-Calcium Pantothenate, Gm.	*dl*-Calcium Pantothenate, Gm.	Panthenol,® Gm.	Natural Tocopherols, Gm.	Ephynal Acetate,® Gm.	Per Cent of Clearing	Complications of Treatment
1, F. B.	M	10.5	Yes	20	258.0	11.0	3,566.0	250.5	85.2	95	G. I.*
2, D. McP.	M	3	No	36	126.8	.....	787.0	.....	75.1	100	None
3, J. M.	M	10	Yes	17	421.5	.....	1,927.0	119.8	146.0	75	None
4, A. M.	F	6	Yes	2	.....	365.0	.....	.....	15.8	80	None
5, B. T.	F	7	Yes	34	1,051.6	2,120.0	.....	43.3	215.3	85	None
6, W. T.	M	2	Yes	10	149.0	.....	1,161.0	.....	175.9	90	None
7, J. R.	F	1	No	8	.....	77.0	137.0	.....	11.4	75	G. I.*
8, A. Y.	F	7	Yes	10	.....	.....	1,330.5	126.4	.....	100	None
9, C. B.	F	7	No	18	423.0	.....	.....	72.5	.....	100	None

* Transient gastric distress from panthenol.®

TABLE 3.—*Treatment of Patients Who Had Subacute Disseminate Lupus Erythematosus*

Case No. and Initials	Sex	Duration of Disease, Yr.	Previous Treatment	Duration of This Treatment, Mo.	*d*-Calcium Pantothenate, Gm.	*dl*-Calcium Pantothenate, Gm.	Panthenol,® Gm.	Natural Tocopherols, Gm.	Ephynal Acetate,® Gm.	Per Cent of Clearing	Complications of Treatment
1, M. A.	F	0.5	Yes	12	.....	240.0	1,928.0	.....	18.3	100	None
2, M. G.	F	1.5	No	22	.....	.....	1,820.5	.....	149.2	95	None
3, B. H.	F	28	Yes	12	30.0	1,917.0	269.0	14.1	213.4	95	None
4, O. L.	F	6	Yes	27	1,673.0	.....	816.0	254.8	70.4	95	None
5, H. T.	F	0.5	No	8	.....	200.0	1,701.0	.....	130.8	99	None
6, V. D.	F	8	Yes	5	199.8	.....	.....	.....	18.0	100	None

one or more of the pantothenic acid radicals. However, such lesions were short-lived and faded out before existing older lesions were completely controlled. Arthralgias developed in six patients while under the above treatment. In each of these patients response of the arthralgia was prompt when sodium salicylate was added to the treatment schedule. In each patient exhibiting increased sedimentation rate, reversed ratio of albumin to globulin, false-positive serologic reactions, leukopenia, relative or absolute lymphocytosis, or albuminuria, reversion of these factors to normal was produced after a variable interval with the above treatment program.

It will be noted in the tables that improvement is recorded in all of the patients who were treated by the combined use of vitamin E and one or more of the pantothenic acid derivatives. Some recovered completely. Many of the others are nearly completely recovered and still under treatment. The response to treat-

ment was best and most rapid in the group with the subacute disseminate type. The group with the disseminate discoid type was next, and that with the chronic discoid type was poorest in response. In the group with the subacute disseminate type improvement was usually noted in three weeks; in the group with the disseminate discoid type, in two months, and in the group with the chronic discoid type, in four months.

Not included in the tables were eight patients with lupus erythematosus (six with chronic discoid, one with disseminate discoid, and one with subacute disseminate lupus erythematosus) who were treated with *d*-calcium pantothenate alone. The dose varied from 2 to 3 gm. daily. There was improvement, but no instance of complete recovery in any patient except the one who had the subacute disseminate type.

COMPLICATIONS ARISING FROM TREATMENT

There were no untoward complications arising from this treatment program except for transient nausea or other slight gastric upset. These symptoms occurred in only a few patients, and usually from the panthenol.* In these patients, either *dl*- or *d*-calcium pantothenate was then used instead of panthenol.* No patient was found who could not take, without reaction, one of the three pantothenic acid derivatives studied. No abnormalities in blood cells, blood chemistry, urine, or blood pressure were noted in patients who were kept on the above treatment program for long intervals. *d*-Calcium pantothenate alone was given to several hundred patients who had various dermatoses, in dosages varying from 0.3 gm. to 3 gm. daily over long intervals without any untoward complications.

Panthenol,* or *dl*-calcium pantothenate, or d-calcium pantothenate together with natural tocopherols or ephynal acetate* was given to 90 patients who had cutaneous diseases other than lupus erythematosus. The number of patients and the dermatoses comprising this group included the following:

40 psoriasis
7 dermatitis herpetiformis
6 lichen planus
6 benign transitory plaques of the tongue
5 chronic infectious eczematoid dermatitis
3 seborrheic dermatitis
3 alopecia areata
2 leukoplakia
2 neurodermatitis

Besides these, one patient was chosen from each of the following disease groups: periarteritis nodosum, vitiligo, contact dermatitis, ichthyosis, morphea, atopic dermatitis, scleroderma, chronic benign pemphigus, prurigo nodularis, epidermolysis bullosum, erythema multiforme, progressive pigmentary dermatosis, erythema perstans, aphthous stomatitis, necrobiosis lipoidica diabeticorum, and lichen sclerosis et atrophicus. These drugs were given to these patients in various combinations and dosages. In no instance was there any complication except for transient gastric distress from panthenol.*

It is evident in the tables that each of 15 patients took over 2,000 gm. (between 4 and 5 lb.), and that Patient 1 in Table 2 took over 8 lb. (3.6 kg.) of these pantothenic acid derivatives without untoward effect. The tables also record 11 patients each of whom took over 200 gm. of vitamin E and Patient 3 in Table 1 who took 424.2 gm. without complication.

SUMMARY

Analysis is recorded of the response to treatment of patients who had chronic discoid lupus erythematosus, disseminate discoid lupus erythematosus, and subacute disseminate lupus erythematosus by the combined use of some forms of vitamin E and one or more derivatives of pantothenic acid. Very favorable results were obtained. There were no harmful effects from treatment noted in this study.

CONCLUSIONS

I feel that the combined use, in adequate dosage, of some form of vitamin E and one or more of the derivatives of pantothenic acid here employed constitutes a safe and effective method of treatment for patients with chronic discoid, disseminate discoid, or subacute disseminate lupus erythematosus.

1219 Carew Tower.

ABSTRACT OF DISCUSSION

DR. ALBERT R. MCFARLAND, Rochester, N. Y.: I would like to discuss Dr. Welsh's very fine paper from the point of view of his concept of the mode of action of these vitamins on lupus erythematosus.

As I think back some 30 years in the past, my first impression was that lupus erythematosus was in the nature of a tuberculid. During my early days of association with Dr. John H. Stokes, he summarized his impression as that of an allergic reaction due either to a tuberculous focus or to a pyogenic focus. Following this concept, we went through a period of cautious but thorough eradication of foci of infection and a search for latent foci of tuberculosis, and also the routine use of gold salts.

Next came the period of stress upon antibacterial agents and the use of arsenic and bismuth. You will recall the paper read before this association by Dr. Richard Weiss on the use of oxyphenarsine (mapharsen®) hydrochloride. A little later Dr. Burgess reported improvement in this disease by the use of large doses of vitamin E. Some other investigators were unable to duplicate Dr. Burgess' findings. An unpublished but very interesting group of cases was presented a few years ago before the general staff meeting of the Rochester General Hospital by Dr. Milton Bohrod. Dr. Bohrod presented several cases with autopsy reports in which there was a combination of periarteritis nodosa, scleroderma, and lupus erythematosus. During life all these patients complained of a great variety of symptoms, and physical findings including foci of infection, allergic conditions, disturbances of liver functions, and thyroid dysfunction. He concluded that perhaps all three of the conditions mentioned were related and might be allergic manifestations of a great variety of different pathological conditions.

Then we come to the era of corticotropin (ACTH) and cortisone. Although neither of these drugs is curative in lupus erythematosus, there is no question but that they have some influence on the course of the disease.

This brings us to the question as to what is Dr. Welsh's theory regarding the action of these vitamins. Do they simply increase the body's resistance against infection? Do they in some way influence allergic reaction? Do they perhaps alter the body's reaction to its own metabolic products, or do they have some indirect effect on the glandular system, such as the adrenals and pituitary? Perhaps it would be too much to ask of Dr. Welsh to give a complete answer to these questions, but it would be interesting to know his line of thought, or, in other words, why he picked this particular line of treatment.

The tables showing the percentage of results obtained are a very important part of Dr. Welsh's paper. The percentage of cure is much higher than by any other method we have used in the past. As I recall, many of his patients obtained 98 to 100% improvement, and I believe the lowest percentage was about 75%.

If this work can be corroborated by other investigators, and if this high percentage of cure can be obtained consistently, it will certainly be a big advance in the treatment of lupus erythematosus.

DR. CARL W. LAYMON, Minneapolis: I think that most of us are far from satisfied with our results in the treatment of lupus erythematosus. Dr. Welsh's remarks were directed toward the chronic discoid and subacute forms of the disease, and he did not deal with the acute septic or the protracted systemic types. Most of us will agree that bed rest is of distinct value in all stages of the disease. This, however, is a most difficult thing to carry out when the patient is not particularly ill. Gold, and even bismuth, in the opinion of many of us, has capabilities of doing great harm in some cases. I think that these drugs have been rather overrated in the treatment of lupus erythematosus. The treatment which Dr. Welsh utilizes apparently has safety, which is extremely important. In closing I wish that he would mention the cost of such treatment.

DR. FRANK C. COMBES, New York: Dr. Welsh has taken a new approach to the treatment of lupus erythematosus. I think it is logical and can be applied to other toxic dermatoses, especially those of the bullous type. All the dermatoses in this entire group are merely cutaneous responses to remote bacterial infections, many of which are situated in the gastrointestinal and upper respiratory tracts.

I think Dr. Welsh should be congratulated for persisting in the administration of the vitamin fractions for so long in the absence of any clinical improvement. It is interesting to philosophize on why it is necessary to administer calcium pantothenate and potassium *p*-aminobenzoic acid for two or three months before benefits are evident. I have had similar experiences, and my ideas are as follows: In all our bodies we have friendly bacteria, which we call saprophytes, and those that eventually cause us trouble and which will eventually get us all some day, which we call pathogens. There is normally a balance established between these groups. We call that "good health." We know that all bacteria require vitamins for their growth and well-being. Of course, some organisms are capable of synthesizing vitamins. This is called autosynthesis, and little is known about it. In infectious diseases and bacterial toxemias the pathogens gain ascendency over both the friendly saprophytes and the immunologic abilities of the host. Pantothenic acid as its name indicates, is an all-inclusive vitamin. Its name is derived from the Greek *pas* (all) plus *theos* (god). In other words, it is "all things to all vitamins." *p*-Aminobenzoic acid occupies a similar but not quite so important position in human nutrition. Both are the protectors of all other vitamins and possibly of unknown enzymes and trace substances necessary for life.

Now when we give these large doses of panthenol* or calcium pantothenate, we are aiding vitamin synthesis in the body, necessary for nutrition of the host, and growth of the friendly saprophytes which slowly increase in number until they are predominant. I have done some studies along these lines which are quite significant but not definite enough to draw conclusions. This method of therapy does cause changes in the bacterial flora of the bowel. It is certainly a valuable addition to our therapeutic approach to the toxic bullous dermatoses and lupus erythematosus.

DR. JACOB H. SWARTZ, Boston: My associate, Dr. Glicklich, and I have used large doses of calcium pantothenate and panthenol* in a small series of 13 patients with lupus erythematosus for the past two and one-half years. Nine of the 13 patients showed fair to good improvement. None were cured. Calcium pantothenate was found more effective than panthenol.* The dose required to obtain beneficial therapeutic results was 6 gm. daily. However, 3 gm. a day was sufficient as a maintenance dose. The results were more striking in subacute and disseminate chronic discoid lupus erythematosus.

We have encountered no significant untoward reactions even in those patients who have been treated with the drug for two years. The addition of vitamin E, not synthetic, did not produce any strikingly better therapeutic results. Recently we tried large doses of ascorbic acid, 2 gm. daily, in combination with calcium pantothenate. Ascorbic acid was tried because of the favorable therapeutic results reported by Massell in rheumatic fever (personal communication). Our series is too small to merit evaluation.

One young female patient with acute disseminated lupus erythematosus improved coincidentally with the administration of large doses of calcium pantothenate. Treatment was then reduced to a maintenance dose of 3 to 4 gm. daily. She had two relapses coincidental with the omission of the drug and improved when calcium pantothenate was resumed. Of course, the report of one case is only important when it serves as an addition to a larger series. One must be cautious in such evaluations.

We have tried calcium pantothenate for other dermatoses, including psoriasis, seborrheic dermatitis, atopic dermatitis, and for two patients with lichen planopilaris. Of 25 patients with psoriasis, 13 showed some questionable beneficial therapeutic results. The two patients with lichen planopilaris showed surprising improvement. We have encountered no beneficial effect from calcium pantothenate in the other dermatoses treated, including one patient with dermatomyositis.

DR. ASHTON L. WELSH, Cincinnati: I am grateful for the ideas for further investigation suggested by my discussors. Some of the things they mentioned are already being studied. The work is still going on, as I indicated in saying that the patients were still under treatment.

I cannot explain the mechanism of the action of the combined use of these drugs in patients who have lupus erythematosus. The theory which Dr. Combes just outlined has, in my opinion, great merit. These drugs have no direct antibacterial action; hence their effect must be produced through some action favoring the activity of the normal defense mechanisms of the body.

The work was started because we had a group of patients at the Cincinnati General Hospital and in our office practice who had had lupus erythematosus for a long time in spite of much treatment with gold, bismuth, liver, etc. Many of them had become reactive and could not tolerate these conventional procedures any longer. A search was made for some approach to treatment of this group.

Pantothenic acid, as Dr. Combes stated, seemed, if one intended to employ any vitamin at all, to be the logical choice because of its wide enzyme-protective action and its pansynergetic action on many other vitamins. Vitamin E was credited with so many separate and unrelated actions, on so many tissues, that the combination of these two agents seemed a reasonable starting combination. In addition, there were favorable reports on the use of some derivatives of pantothenic acid in patients who had lupus erythematosus, and also reports of improvement from the use of vitamin E in patients who had this disease.

One drug company was willing to give us enough of these materials to start this experiment. After we had begun the study, because of the cost of the agents used, it became necessary to interest another company also in supplying the materials, in order to get adequate supplies. Patients were switched from one company's materials to materials of another company, depending on the materials currently available. Accordingly, most of the patients had combinations of several types of the two agents used. Later, it was possible to get these materials through our own pharmacist. He was able to supply these agents, at fairly reasonable prices, by purchasing very large quantities from the manufacturers, thereby obtaining a lower price.

The high dosages of the drugs employed and the long treatment intervals were arrived at automatically. As long as the patients had lesions, the drugs were administered to them, and whenever the patients seemed to cease to progress favorably, the dose was elevated. Gradually high dosage levels were reached. It was noted that each time we did elevate the dosage, the patients again showed improvement. As long as these drugs did not produce any sign of toxic reaction, no reason for not using them in this way presented itself.

I was interested in Dr. Swartz's report of his experience with the use of calcium pantothenate and panthenol® for patients who had lupus erythematosus, but I feel that his results would have been much better had he used higher dosages. I feel that his observation that the addition of natural vitamin E did not appreciably improve the therapeutic results was due to the fact that only small quantities of vitamin E were used. I was gratified that his patients improved, as did ours, on calcium pantothenate and panthenol,* and that he attested to the safety of administration of these medications.

Dr. Laymon expressed my own feelings regarding the use of this approach in handling lupus erythematosus over the older, conventional procedures on the basis of safety of the drugs employed.

The work is still continuing. An attempt is being made to find the most effective salt of pantothenic acid in the treatment of lupus erythematosus, and also to find what form of vitamin E produces the best therapeutic result when combined with that salt. It appears, to date, that the dextrorotatory form of the calcium salt of pantothenic acid and the dextrorotatory form of the acetate ester of the alpha tocopherol constitute the most effective combination yet employed.

While these agents are expensive, I do not think they are beyond purchase by many patients. Many patients now under treatment are purchasing their own materials. I do not know the exact cost, at the moment.

LUPUS ERYTHEMATOSUS

Treatment by Combined Use of Massive Amounts of Panthothenic Acid and Vitamin E

ASHTON L. WELSH, M.D.
CINCINNATI

THIS REPORT deals with rationalization for the use of, and results obtained in the treatment of, certain phases of lupus erythematosus by the administration of massive amounts of pantothenic acid (calcium and sodium salts, and the alcohol thereof) and vitamin E. Patients who had chronic discoid, disseminated discoid, and subacute disseminated lupus erythematosus were studied. Maintenance therapy of patients with acute disseminated lupus erythematosus controlled by steroid hormones was likewise studied. Results were reported on the basis of preliminary study in 1951.[1] Confirmation of these results after further study and additional data follow.

THERAPEUTIC AGENTS EMPLOYED

PANTOTHENIC ACID

Pantothenyl alcohol (Panthenol) *	consisting of the alcohol of pantothenic acid, which is, chemically, *d*-α, γ-dihydroxy-N (3-hydroxypropyl) β, β-dimethylbutyramide.
Calcium pantothenate, dextrorotatory †	consisting of the calcium salt of pantothenic acid, the molecule of which is composed of β, β-dimethyl-α-hydroxybutyroactone condensed with β-alanine.
Calcium pantothenate, racemic ‡	consisting of the calcium salts composed of an equal number of dextro- and levo-rotatory constituents.
Sodium pantothenate, dextrorotatory §	consisting of the sodium salt of pantothenic acid.

VITAMIN E

The following forms of vitamin E were used in this study:

Ephynal acetate ‖	consisting of *dl*-α-tocopherol acetate (synthetic vitamin E acetate).

Read at the meeting of the American Dermatological Association, Lake Placid, N. Y., June 11, 1953.

* Panthenol for study was supplied by Hoffman-LaRoche, Inc.

† Calcium pantothenate, dextrorotatory, for this study was supplied by Lederle Laboratories Division, American Cyanamid Company; Merck & Company, Inc.; National Oil Products Company; Chas. Pfizer & Company, Inc.

‡ Calcium pantothenate, racemic, for this study was supplied by Abbott Laboratories.

§ Sodium pantothenate, dextrorotatory, for this study was supplied by Parke, Davis & Company.

‖ Ephynal acetate for this study was supplied by Hoffmann-LaRoche, Inc.

Natopherol acetate ¶	consisting of vitamin E concentrates containing *d*-α-tocopherol acetate with related tocopherol acetates. Each 100 mg. contains 73 mg. of *d*-α-tocopherol acetate, equivalent to 100 mg. of *dl*-α-tocopherol, by rat antisterility assay comparing the activities of *d*-α-tocopherol and *dl*-α-tocopherol; or equivalent to *d*-α-tocopherol 108 mg. by rat antisterility assay, comparing the activities of *d*-α-tocopherol and *d*-α-tocopheryl acetate.
Aquasol E ‖	consisting of a water-soluble form of *d*-α-tocopheryl acetate, equivalent in vitamin E biological activity to 100 mg. of *dl*-α-tocopherol (oil-soluble vitamin E made water-soluble with sorethytan esters).

RATIONALIZATION FOR THE USE OF PANTOTHENIC ACID COMBINED WITH VITAMIN E

Pantothenic Acid

Much research has been done (and continues) to establish the role of pantothenate as a vitamin and the metabolic functions of pantothenic acid.[2] Symptoms of nutritional deficiency of pantothenate reveal "only the lack of a substance fundamental to cellular physiology in general could cause such diverse symptoms."[3]

It is fairly well established, now, that as nutritional deficiency of pantothenate progresses, certain vital metabolic processes slow down and cease. This will result in incomplete synthesis of functional enzymes and coenzymes, which will have the effect of causing deficiencies of other factors.

Some of the more important manifestations of pantothenate deficiencies are the following:

1. Lesions of the skin and hair.*
2. The storage of riboflavin.*
3. Gastrointestinal symptoms.†
4. Lesions of the nervous system ‡
5. Inhibition of antibody formation (pantothenate and pyridoxine deficiency leads to severe impairment of antibody production) §
6. Lesions of the adrenal gland

Observations seem to demonstrate that in pantothenate deficiency the adrenal cortex is depleted of hormone, although there are opposing views explaining the cause of this adrenal change. One of these views suggests that adrenal changes reflect functional insufficiency of the gland due to inability to synthesize hormone, implying that in some manner, pantothenate is directly concerned with hormone synthesis.‖

Evidence suggests functional inadequacy of the adrenal cortex during pantothenate deficiency.[21] Pantothenate-deficient animals have a lowered fasting blood

¶ Natopherol Acetate for this study was supplied by Abbott Laboratories, and Distillation Products Industries, Division of Eastman Kodak Company, kindness of Dr. Norris Embree

‖ Aquasol E was supplied by U. S. Vitamin Corporation.

* References 4 to 8.

† References 10 to 12.

‡ References 13 and 2.

§ References 14 and 16.

‖ References 17 to 20.

sugar and increased sensitivity to insulin, both observations being consistent with adrenal insufficiency. In the vitamin-deficient state, deficient animals are unable to respond to epinephrine or corticotropin (ACTH) by lowering eosinophiles.¶

The injection of cortisone causes the deficient animals to behave like normal animals in eosinophile response. The fact that deficient animals respond like normal animals to cortisone is taken to indicate that the mechanism of response is intact but the animals are unable to elaborate the hormone.

Changes which the adrenal gland undergoes during pantothenate deficiency show that pantothenate is involved in the synthesis of adrenal cortex hormones. In pantothenic acid deficiency there develops a definite insufficiency of adrenocortical function.[24]

According to Cowgill: "Pantothenic acid is needed in the chemical process by which the adrenal gland produces certain of its hormones, and may be considered a biological catalyst."#

In 1950, Maas and his co-workers[27] showed that Escherichia coli actually excretes pantothenate.

It is recognized that pantothenate exists in tissues in bound form. In 1945, discovery was made of a coenzyme, coenzyme A (CoA),* of which pantothenate is a constituent. It appears that pantothenic acid, probably acting through coenzyme A, is necessary for the biosynthesis of the adrenal steroids.[28]

Nutritional deficiency of pantothenic acid leading to greatly reduced coenzyme A levels in tissues would adversely affect many other reactions. Synthesis of acetylcholine would be deranged; likewise, the acetylation of glucosamine and galactosamine would be reduced.[3] Since these compounds are components of connective tissue, there may result abnormalities of this type of tissue.

It has been emphasized that lesions of the chronic discoid and acute types of lupus erythematosus are pathologically alike, and, as Klemperer has so ably pointed out, the common denominator is the connective tissue.[30]

Continuing and advancing research for knowledge about pantothenate and enzymatic steps in its conversion are indicated by announcement of the synthesis of pantothine, reported at the most recent meeting of the American Chemical Society.[31]

It is concluded from the foregoing that in pantothenic acid deficiency there is an insufficiency of adrenocortical function.

Vitamin E

The term "vitamin E" has been applied to α-tocopherol, and, to a less extent, to the β, γ, and δ tocopherols, and certain synthetic compounds.

The deficiency state experimentally (or accidentally) induced in laboratory and domesticated animals is characterized by manifestations varying from encephalomalacia and exudative diathesis in the chick, to fetal absorption, testicular damage, muscular dystrophy, and other effects in mammals.†

¶ References 22 and 23.
# References 25 and 26.
* References 28 and 29.
† References 32 to 40.

The serum tocopherol levels in normal adults and in those suffering from disease have been extensively studied.‡ Such studies indicate that the mean serum level of tocopherol is less than normal in malnourished persons, as well as in persons with disease states, particularly those associated with impaired absorption of fats.§

The tocopherols, likewise, possess the power to augment other vitamins. Individuality of vitamin E, the modifying agent, may not be evidenced, but it undoubtedly influences the physiological activity of other vitamins. This phenomenon has been described as "sparing" or "synergistic" and has lead to application of the term "covitamin E" activity.‖

The tocopherols are phenolic antioxidants. In general, any acid-type inhibitor will promote or energize the activity of any phenolic antioxidant. Ascorbic acid (vitamin C) enhances the antioxygenic activity of the tocopherols.[57] In addition, vitamin C, because of its marked reducing activity, protects vitamin E in the intestinal tract.[58]

Vitamin E protects biotin and other members of the vitamin B complex against destruction by rancid fats in the intestinal tract.[59] Vitamin E also protects against phosphorus turnover.[60]

SUMMARY OF INVESTIGATIVE STUDIES

Vitamin E and Pantothenic Acid Derivatives Used Individually in the Treatment of Lupus Erythematosus

Klemperer's study of the pathology of disseminated lupus erythematosus in relation to involvement of various organs described skin changes [61] as homogenization of collagen fibrillae and ground substance, with concomitant proliferation of fibroblasts and degenerative "stigmas," which seemed to indicate involvement of connective tissue. Vascular changes in the skin and elsewhere follow a pattern of connective tissue involvement.

Vitamin E.—Burgess,[¶] observing that tocopherols in sufficient dosage demonstrated ability to regenerate collagenous tissue in a case of noduloulcerative granuloma with lipoidal deposits indistinguishable from necrobiosis lipoidica diabeticorum, and, reasoning that lupus erythematosus affected primarily collagenous ground substance, studied mixed tocopherol therapy in the treatment of lupus erythematosus. He reported favorable results after prolonged therapy followed by maintenance therapy after clinical cure.

Sweet [64] failed in attempted confirmation of Burgess' results on tocopherol therapy. Sawicky [65] likewise reported failure. Morgan [66] agreed with Sweet. A group of investigators at New York University Hospital [67] studied tocopherol therapy for discoid lupus erythematosus and other dermatoses, using both oral and injectable materials, and confirmed Morgan's results.

Study of the effectiveness of tocopherols (in keeping with the ideas of Burgess and Pritchard) during a period of 14 months was undertaken on 60 cases of localized and disseminated discoid types. "Real and effective" action was reported on 15 patients. The large proportion of failures was attributed to insufficiency of the

‡ References 41 and 42.
§ References 43 to 48.
‖ References 49 to 56.
¶ References 62 and 63.

dosages utilized.[68] Vitamin E administered orally, daily, produced definite amelioration but not absolute cure of chronic lupus erythematosus.[69] Vitamin E induced complete cure of poikilodermia and lupus erythematosus; the latter recurred and was again "cured" with tocopherol therapy.[70]

Of a series of 25 patients with lupus erythematosus treated by oral administration of vitamin E, 17 recovered but 2 relapsed within six months after cessation of treatment. Four patients showed improvement: four became worse.[71] Successful management with vitamin E was reported in 10 cases of chronic lupus erythematosus which had previously responded to other therapy and then relapsed.[72] Several cases of lupus erythematosus were treated with vitamin E with beneficial results. Erythema and infiltration diminished in a few weeks and cleared completely after a few months with tocopherol therapy.[73]

Pantothenic Acid.—Goldman [74] reported results of treatment of subacute discoid lupus erythematosus with calcium pantothenate. He concluded that calcium pantothenate offered possibilities as the least toxic of available chemotherapeutic agents for prolonged therapy. He followed his first report with a favorable preliminary study on intensive pantothenyl alcohol therapy.# Cochrane and Leslie [77] failed in attempted confirmation of Goldman's results.

ANALYSIS OF CLINICAL DATA

Sixty-seven patients who had lupus erythematosus (diagnosis confirmed by biopsy) were treated with massive-dose therapy consisting of pantothenic acid derivatives combined with vitamin E. Patients are classified according to the disease phase presented.

No. of Patients	Disease Phase
36	Chronic discoid
17	Disseminated discoid
11	Subacute disseminated
3	Acute disseminated

Twenty-eight of this group had had no previous therapy. Thirty-nine had had previous treatment, consisting of prolonged and diversified types of therapy including gold, bismuth, arsenic compounds, liver, salicylates, and steroid hormones. All of these patients improved.

Chronic Discoid Group.—This group was comprised of 36 patients.

No. of Patients	Cleared to Date, %
15	100
6	95
6	90
3	80
3	75
3	60-70 *

* Therapy started too recently for adequate appraisal of response.

Patients in this group had had the disease for time intervals of 10 to 30 years. Considerable amounts of scarring were observed, on original examination, among those patients who had had previous therapy with solid carbon dioxide, x-ray,

References 75 and 76.

radium, or acid. Most of the lesions presented were markedly infiltrated and hypertrophic. Nine patients had experienced episodes of the disseminated discoid phase of the disease.

Objective improvement in the average patient, in this group, began after (approximately) four months, after (approximately) six months for those patients with lesions of many years' duration and marked hypertrophy.

Arthralgia developed in 10 patients while under treatment. Response to the addition of sodium salicylate in eight of these patients was prompt. In the other two patients, response occurred after removal of dental foci.

Eighteen additional patients with chronic discoid lupus erythematosus began treatment with massive-dose therapy consisting of pantothenic acid derivatives combined with vitamin E. Most of them showed some improvement, but all of them disappeared from observation after one or two months.

Disseminated Discoid Group.—This group was comprised of 17 patients.

No. of Patients	Cleared to Date, %
7	100
4	95
2	90
4	80 †

† Therapy started too recently for adequate appraisal of response.

Objective improvement was noted after (approximately) two months.

Subacute Disseminated Group.—This group was comprised of 11 patients.

No. of Patients	Cleared to Date, %
9	100
2	75 †

† Therapy started too recently for adequate appraisal of response.

Response to therapy was most rapid, and best, among these patients, and was manifested (usually) in one month.

Acute Disseminated Group.—This group was comprised of three patients. These patients were started on massive-dose therapy of the agents under study while the acute phase of the disease was being brought under control with steroid hormones. These patients have been maintained on massive-dose therapy without relapse, for time intervals as follows: 7 months, 11 months, and 19 months, respectively.

In general, the more hypertrophic and infiltrated the process, the slower the response. It has been necessary to persist with therapy for as long as three years in some patients.

In some patients therapy was not continuous but was interrupted for various reasons. In those patients, progress ceased, and if therapy stopped for long, relapse occurred, or new lesions developed. Eleven patients developed new lesions while undergoing therapy, but such lesions were short-lived and usually faded out before existing older lesions were completely controlled. It was possible to maintain maximum dosage schedule, for some patients, only during the last four months of treatment course.

In all patients not yet completely cleared, improvement has been marked. Patients not cleared entirely are still under treatment.

After clearing, the lesions exhibited minimal residual atrophy and pigment disturbance. Even the old scars of previously treated, inactive lesions flattened.

smoothed out, and lost most of the telangiectasia. These changes in old scars may have resulted from the action of prolonged use of vitamin E on the collagen and fibrous tissues in the lesions and blood vessels.

Patients who had maintained the massive-dose therapy program for some months regularly volunteered the information that they "felt better," or that they "had a sense of well-being." This feeling had no relation to objective change in the skin lesions. This reaction simulated the euphoria experienced by some patients taking cortisone.

TREATMENT PROGRAM

Early in this study, patients were treated with combined use of relatively small amounts of one of the pantothenic acid derivatives and one of the vitamin E preparations. Later, when it became apparent that higher dosages were required, the following schedule was employed:

Pantothenic acid

Calcium pantothenate	10-15 gm. daily
Pantothenyl alcohol	10-15 gm. daily
Sodium pantothenate	5-10 gm. daily

(The nature of this light crystalline substance permitted inclusion of only 0.25 gm. per capsule. Difficulty presented by daily number of required capsules necessitated use of smaller amounts of this agent.)

Vitamin E

Ephynal acetate	1,000-2,000 mg. daily
Natopherol	1,000-2,000 mg. daily
Aquasol E	1,000-2,000 mg. daily

COMPLICATIONS ARISING FROM THERAPY

As has been previously reported,[1] there were no complications arising from this treatment program except for transient nausea and gastric distress. It was previously reported that such distress occurred chiefly with pantothenyl alcohol, but as the present study progressed, it was found that transient nausea and gastric distress occurred about equally from use of calcium pantothenate, sodium pantothenate and pantothenyl alcohol. Symptoms of such distress occurred in only a few patients, and no patient was found who could not take one of the pantothenic acid derivatives studied.

No abnormalities were revealed by repeated hematological studies and urinalyses of patients kept on this program of therapy for intervals varying from one to three years.

Varying combinations and massive dosages of pantothenic acid derivatives and vitamin E preparations were given to 154 patients who had diseases other than lupus erythematosus. There was no complication, except for gastric distress abovementioned.

LABORATORY DATA

URINARY EXCRETION LEVELS—PANTOTHENIC ACID DERIVATIVES‡ (MORNING URINE SPECIMENS)

Average level before start of therapy in patients with lupus erythematosus was 24.24 γ/ml. Five patients had levels averaging somewhat higher than the others. It was noted, however, that these patients had been taking large quantities of multiple-vitamin preparations containing pantothenic acid, vitamins A, C, E, and components of the vitamin B complex, including riboflavin, inositol and pyridoxine.

‡ These studies were done by Hill Top Research Institute, Inc., Cincinnati.

Average level of normal persons used as controls was 2.69 γ/ml.

Average levels of patients under treatment were as follows:

Oral Daily Dosage, Gm.	Calcium Pantothenate γ, Ml.	Pantothenyl Alcohol (Panthenol) γ, Ml.
5	1,843.2	2,891.1
7½	3,624.3	5,688.4
10	4,084.8	5,782.7
12½	5,698.8	13,144.1
15	15,730.0	15,945.0

Levels higher than those for calcium pantothenate, but not so high as those for pantothenyl alcohol at the same dosages, were obtained from patients on sodium pantothenate. Because of the bulk of this material, however, the agent was impractical for use in this study.

The increase in excretion of pantothenic acid (over that of normal persons) in patients with lupus erythematosus before start of therapy may be indicative of some metabolic fault in patients with lupus erythematosus.

The increased urinary excretion levels of pantothenic acid exhibited with increase in dosage levels of pantothenic acid derivatives justifies the dosages employed and may account for the more rapid clinical improvement on the higher dosages.

For this study, the method of Hoag, modified to include use of pantothenate assay medium, dehydrated Difco No. B-323 as the test medium, was employed.§

In order to obtain greater accuracy, determinations of pantothenic acid blood levels instead of urinary excretion levels have recently been undertaken. The findings parallel closely the urinary excretion levels, but show less variation.

Vitamin E Blood Levels ‖

Average level before start of therapy in patients with lupus erythematosus was 0.567 γ/100 ml. serum

Six patients had levels averaging 2.060 γ/100 ml. serum but it should be noted that these patients had been taking large quantities of multiple-vitamin preparations containing vitamins A, C, E, and components of the vitamin B complex, including folic acid, inositol, and B_{12}. High vitamin E levels may have been due to ingestion of vitamin C, because of the latter's reducing action which protects vitamin E.

Average level of normal persons used as controls was 2.370 γ/100 ml. serum.

Average levels of patients under treatment were as follows:

Oral Daily Vitamin E-All Types, Dosage, Mg.	Average Blood Level γ, 100 Ml.
300	2.30
1,000	4.20
1,500	5.56
2,000	6.69

Effort was made to determine the average blood levels (expressed as mg./100 ml. serum) produced by each of the oral vitamin E preparations available for this study.

Dosage, Mg.	Ephynal	Natopherol	Aquasol E
300	2.54	2.66	3.11
1,000	3.87	4.66	5.27
1,500	5.66	5.17	6.20
2,000	5.67	5.87	7.12

Vitamin E preparations for parenteral administration were given weekly, because it was impossible to get patients in for treatment oftener than at weekly intervals. All parenterally administered preparations produced significant rises in vitamin E blood levels. Supplementation

§ References 78 and 79.

‖ Early studies were done by Dr. L. J. Filer Jr., Rochester, N. Y. Later studies were done by Hill Top Research Institute, Inc., Cincinnati.

of oral administration, with parenteral administration, appeared to speed up response in recalcitrant patients on oral massive-dose therapy of vitamin E, combined with one of the pantothenic acid derivatives. Additional studies are being undertaken with materials for parenteral administration.

Patients with lupus erythematosus were shown, by laboratory data, to have vitamin E blood levels lower than normal. The increase in blood levels with the increase in dosage of vitamin E, justifies use of massive-dose therapy, and may explain the more rapid rate of favorable clinical response obtained at the higher dosage levels.

For this study, the micromethod of Quaife was modified so that ordinary 10 mm. cuvettes might be employed.[80]

Carotene Levels ¶

Average level before start of therapy in patients with lupus erythematosus was 75.6 γ/100 ml. serum. (This is lower than the reported normal levels of 169.0[81] and 166.0 for men, and 187.0 for women.)[82]

Average level for normal persons (in our laboratory) was 142.4 γ/100 ml. serum.

Levels rose, with vitamin E levels, in patients with lupus erythematosus under treatment with massive-dose therapy of Vitamin E combined with one of the pantothenic acid derivatives.

COMMENT

The foregoing clinical data represent the investigator's second report on a series of patients with various phases of lupus erythematosus treated with massive doses of pantothenic acid derivatives and vitamin E. Results of a preliminary study have already been reported.[1]

The work of other investigators using these therapeutic agents individually, rather than in combinations, has been summarized. Results from their studies, for the most part, were unfavorable.

The following postulation is offered to explain why use of either of the individual agents, alone, is a therapeutic failure, and why use of the combined agents produces favorable response.

1. Pantothenic acid, a B vitamin, is needed in the chemical process by which the adrenal gland produces certain of its hormones.

2. All four known tocopherols (α, β, γ, δ) contain a phenolic hydroxyl group, the structural function of which is the potential oxidizability to a *p*-quinonoid form (which is important for antioxidant power).

3. α-Tocopherol may not form a reversible oxidation-reduction system with the *p*-quinone, but there may be intermediate oxidation products, perhaps semiquinones, which could be in equilibrium with tocopherol to furnish the oxidation-reduction mechanism for antioxidant action.

4. If α-tocopherol is oxidized, the *p*-quinone can be produced. The *p*-quinone may give an equilibrium with tocopherylhydroquinone, a trihydroxy compound.

5. Tocoherol and tocopherylquinone have already been identified on the basis of their distribution constants and absorption spectra in extracts of stools collected during control periods and during periods when tocopherol was orally administered, supporting the hypothesis that tocopherol is oxidized to its quinone in the body. Other reducing materials including the carotenes were shown to be present in feces.#

¶ These studies were done by Hill Top Research Institute, Inc., Cincinnati.

References 83 and 84.

6. By oxidation at different points of the cyclopentenophenanthrene nucleus there may be obtained probable precursors of cholic acid, of adrenal cortex hormone and progesterone, and of estrogenic and androgenic hormones.

A sufficient supply of vitamin E should act on the anterior pituitary gland to produce a cholesterol-degrading hormone capable of promoting these oxidations. Such a hormone is reported to have been obtained from the anterior pituitary glands of pregnant guinea pigs previously treated with vitamin E.[45]

7. Under the influence of vitamin E, the production of the hormone in the anterior pituitary gland is stimulated and its action is promoted so that normal dehydrogenation of cholesterol is encouraged in the human body and its abnormal dehydrogenation to carcinogenic compounds is prevented.[55]

8. Furthermore, by means of the Thorn test (decrease of eosinophiles in blood) and of the formalin test (counteraction of the inflammation produced by intracutaneous injection of formalin) vitamin E has been found to have activity similar to adrenocorticotropic hormone and to cortisone.[56]

The precise interrelationship of the adrenals, the pituitary, and the various tissues of the body is not clearly defined. Moreover, the functions and modes of interplay of the individual steroid hormones produced by the adrenal cortex are extremely complex.

The present concept is that the action of cortisone and other cortical steroid hormones is entirely mediated through stimulation of the adrenal cortex by the adrenocorticotropic hormone. This hormone is elaborated by the anterior pituitary. The function of the anterior pituitary is, presumably, controlled to some degree by the hypothalamus, which, in turn, can be influenced by higher brain centers.

The adrenocorticotropic hormone has the specific function of regulating activity of the adrenal cortex. It is in response to the action of this hormone that the adrenal cortex secretes its steroid hormone.

It is suggested that the metabolic products of vitamin E, such as the quinones, may serve as "building blocks" to aid in the formation of cortisone-like materials, which are presumed to be excreted by the adrenals. Calcium pantothenate may be an additional biological catalyst, along with the adrenocorticotropic hormone to help aid in the formation of new cortisone-like materials. It is necessary to use extremely large quantities of calcium pantothenate as a catalyst because most of it is quickly metabolized in the body.

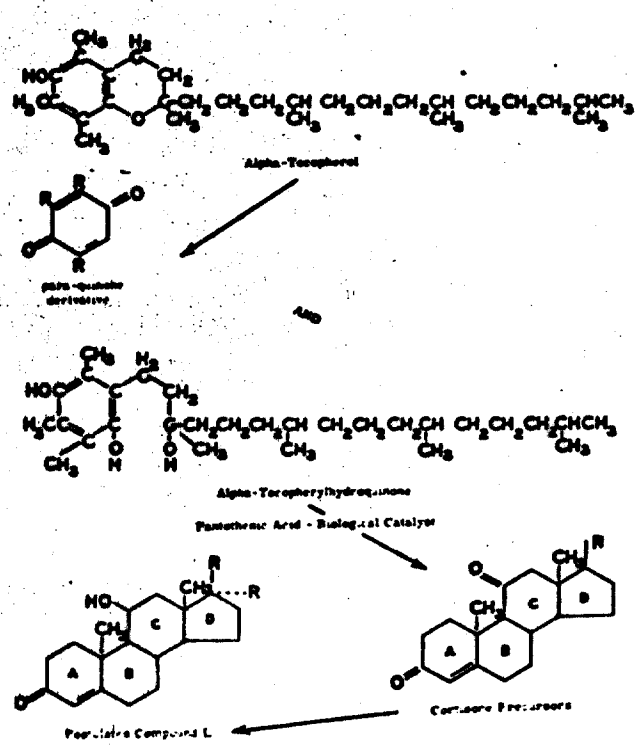
Furthermore, it may be that the combined catlytic activity of the adrenocorticotropic hormone and calcium pantothenate on vitamin E metabolites may produce within the body even more potent cortisone-like bodies than cortisone itself, since it is now known that compounds, such as, 9-α-chloro-17-α-hydroxycortisone have been found to have three to four times the activity of that of cortisone.[57]

During the past few years, much valuable and intensive work has been directed toward clarifying the fundamental mechanism of the action of cortisone against disease. It seems likely that the therapeutic action of cortisone involves not only the many known physiological properties of the hormone but various thus far undis-

covered effects on the chemical processes, organs, tissues, and cells of both normal and diseased persons. In any event, it has not yet been demonstrated that the mode of action of cortisone can be attributed to a single circumscribed mechanism.

It is postulated that cortisone, or some cortisone-like material is perhaps being slowly synthesized within the body from massive-dose therapy of pantothenic acid derivatives combined with vitamin E. What this material is—is not known. For the sake of convenience, and, pending acquisition of additional knowledge, based on further study, this material is designated as "compound L."

Theories, Postulations, and Theoretical Formulations.—The concept relating to results obtained in the treatment of lupus erythematosus by massive-dose therapy



of pantothenic acid derivatives combined with vitamin E is based on the possibility that vitamin E may be metabolized in the body to *p*-quinones and tocopherylhydroquinones, which may aid in the formation of, or furnish the "building blocks" for some precursor cortisone-like materials; additionally, that calcium pantothenate may be intimately related to the function of secretion and formation of steroid hormones known to be products of the adrenal cortex. This theory is supported by the functional inadequacy of the adrenal cortex during calcium pantothenate deficiency.

It is possible that a deficiency of pantothenate in the body can retard production of cortisone and other adrenal hormones. Pantothenic acid, a B vitamin, may be considered a biological catalyst, and, probably acting through coenzyme A, may be

necessary in the chemical synthesis of steroid hormones secreted by the adrenals. It is possible that pantothenic acid, as well as the adrenocorticotropic hormone, aids in the stimulation of the adrenal cortex and the pituitary in the chemical process of production of cortisone or related derivatives.

Vitamin E in the body may be metabolized to α-tocopherylhydroquinone and α-tocopherylquinone, both compounds found (on rabbits) to be more active when injected intravenously than when given by mouth."[8]

Tocopherylquinones and hydroquinones in turn may be metabolized or further modified to some precursor cortisone-like materials. Quinone might be considered a basic starting material for the biologic synthesis of cortisone, and help build up to the skeleton containing the A, B, C and D ring structure.

Diagrammatically, this theory is shown in the accompanying chart.

SUMMARY

A program of therapy, comprising combined use of massive amounts of pantothenic acid derivatives and vitamin E, was employed in the treatment of 67 patients with various phases of lupus erythematosus. Response was most favorable. Reactions to therapy were insignificant.

Rationalization for use of program of therapy is outlined.

Summary is recorded of investigative work, by others, employing pantothenic acid derivatives and vitamin E, individually, rather than in combination, for the treatment of various phases of lupus erythematosus.

Laboratory data is presented in support of massive-amounts dosage schedules.

A theory is postulated to account for favorable action of combined use of pantothenic acid derivatives and vitamin E in the treatment of lupus erythematosus.

CONCLUSIONS

Combined use of massive amounts of pantothenic acid derivatives and vitamin E constitutes a safe and effective approach to treatment of patients with chronic discoid, disseminated discoid, and subacute disseminated lupus erythematosus. Limited experience suggests this procedure as maintenance therapy for patients with acute disseminated lupus erythematosus, the acute phase having been controlled through use of steroid hormones.

ABSTRACT OF DISCUSSION

Dr. Clinton W. Lane, St. Louis: Dr. Welsh is to be congratulated for a thorough, painstaking study of the chemical reactions of pantothenic acid and vitamin E and a complete review of the various theories concerning their modes of activity in the body. He has stated the reasons why he believes they should be effective in the treatment of lupus erythematosus and has produced statistics to show that the therapeutic results prove his opinions to be correct.

Since the cause of lupus erythematosus is unknown, treatment has for the most part been on an empiric basis. However, two therapeutic ideas have prevailed, the one to administer drugs with an anti-infection property, such as gold, bismuth, arsenic, the salicylates, quinine (Atabrine; Mepacrine), and chloroquine (Aralen), the other an attempt to improve the defense mechanism and to boost the resisting powers of the body by the use of liver injections, transfusions, vitamins, adrenocorticotropic hormone and cortisone (Cortone).

Important reasons why Dr. Welsh has chosen to give pantothenic acid are that with a deficiency of this vitamin there is an insufficiency of adrenal cortex hormone and that pantothenate is involved in the synthesis of antibodies. Vitamin E was chosen because it has the power to augment other vitamins and because Welsh agrees with Burgess that tocopherols have the

ability to regenerate collagenous tissue. These appear to be logical and valid reasons, particularly if one agrees that lupus erythematosus pathologically shows primarily a connective tissue involvement. The therapeutic results obtained in chronic and disseminated discoid and subacute disseminated lupus erythematosus are exceptionally good, even though they are much slower and less dramatic than those obtained with quinacrine, cortisone, and gold. The vitamins employed have the added advantage of being nontoxic, even in the high dosages administered for months and years.

The failure of others to obtain the satisfactory results of Welsh, Goldman, and Burgess are traced by these investigators to inadequate dosage, yet several months after Goldman's good results were published, Cochrane and Leslie, using the same dosage of calcium pantothenate and panthenol had 100% failure in 46 patients. Others, and I myself, had similar poor results, and failures were recorded by numerous investigators who attempted to duplicate the fine results obtained by Burgess with vitamin E. It may be that the dosages were inadequate, that not enough time and patience were exercised, and that the proper combination of the two vitamins was not employed.

Dr. Welsh has given vitamin E to patients with lupus erythematosus because the blood level is lower than in normal persons and then gives pantothenic acid because the urinary excretion of pantothenic acid is higher in lupus erythematosus patients than in normal persons. Has he in his additional studies found the blood level of pantothenic acid to be lower than normal and the urinary excretions of vitamin E to be higher than that of normal persons?

The cost of these vitamins in the dosages recommended should be borne in mind. The materials were donated to Dr. Welsh for investigative purposes, but if the patient had to purchase the vitamins in St. Louis in the dosage recommended, the daily cost would vary between $3.00 and $4.50. If this was continued for three years as was necessary in some patients, the total cost of the drugs alone would be almost $5,000. This is a tidy sum even in this golden age when there is little quibbling about the cost of commodities whether desired or necessary.

A correct appraisal seems to be that in these two vitamins we have a safe approach to the therapy for lupus erythematosus and that they should be tried when quinacrine, chloroquine, and more rapidly acting drugs have failed or when toxicity to these drugs has developed. We are indebted to Dr. Welsh for offering us an effective method of therapy in the treatment of a stubborn, chronic dermatosis.

DR. J. H. SWARTZ, Boston: I have had the opportunity of discussing Dr. Welsh's original paper. My associate, Dr. Glicklich and I have used calcium pantothenate in lupus erythematosus in these various dosages mentioned, since Dr. Goldman's paper.

First, a word about the maintenance dose. I have one case in particular that I want to cite, a case of acute disseminated lupus erythematosus, before the cortisone therapy era, who, at least coincidental with the administration of large doses of calcium pantothenate, recovered. Of course, we don't know whether the calcium pantothenate was responsible for the remission except that she relapsed each time the calcium pantothenate was stopped. Cortisone therapy administered during her last relapse resulted in a psychic disturbance. She is now taking calcium pantothenate and is in a remission period.

I have used calcium pantothenate alone, in doses varying from 7 to 12 gm. a day. I have used calcium pantothenate in conjunction with large doses of vitamin C, 500 mg. three times daily, and I have had a series of patients who received calcium pantothenate with vitamin E. My own impression is that the combination of vitamin C and calcium pantothenate does equally as well as vitamin E and calcium pantothenate. I would like to ask Dr. Welsh whether he had similar experience.

It is important that dextrorotatory calcium pantothenate be administered. The racemic calcium pantothenate is not as effective.

It is true that it is very expensive. The Massachusetts General Hospital supplies it at cost for our patients, thus making it easier for them financially. The dose can be gradually cut down. I have cut it down to three 500 mg. capsules three or four times a day, and found it sufficient during a period of remission.

I agree with Dr. Welsh and the previous speaker that the chronic, thickened discoid type does not respond so well, that the subacute disseminate or the generalized discoid type responds best.

I enjoyed Dr. Welsh's paper very much.

DR. CLARENCE S. LIVINGOOD, Detroit: I, too, would like to congratulate Dr. Welsh for carrying on this tedious long-term careful study. In discussing his paper, it is pertinent to emphasize that no one in this country or abroad has used his exact dosage schedule in administering these drugs for the treatment of lupus erythematosus.

We have treated a few patients in almost the same manner that he describes and for as long as 12 to 18 months. However, my discussion must be predicated by the fact that the majority of our patients received pantothenyl alcohol (Panthenol) alone, or a combination of pantothenyl alcohol or calcium pantothenate and α-tocopherol for much shorter periods of time; none of our patients received more than 800 mg. of α-tocopherol daily; the dosage of pantothenyl alcohol or calcium pantothenate was from 10 to 15 gm. daily.

It is agreed that this therapy seems to be of more value for patients who have subacute disseminate lupus erythematosus rather than the chronic discoid variety. No evidence of toxicity or a serious side-effect was noted in our series of patients, although some patients had diarrhea. In a few instances it was necessary to reduce the dose of pantothenyl alcohol or calcium pantothenate for a short period of time, in which case the number of stools decreased significantly.

We feel that this combined pantothenyl alcohol and α-tocopherol treatment described by Dr. Welsh has influenced favorably the course of several of our patients who had subacute disseminated lupus erythematosus. We have not been impressed with the results in the case of patients with chronic discoid lupus erythematosus, although as noted previously, we did not duplicate Dr. Welsh's dosage technique, and in only two cases was the treatment continued for more than one year.

In conclusion, it is my impression that this combined pantothenyl alcohol and α-tocopherol treatment seems to be indicated for some patients who have subacute disseminated lupus erythematosus. In our experience, the therapeutic results seem to be somewhat unpredictable. There are disadvantages involved in administering such treatment which makes it somewhat impractical for the majority of patients.

DR. ASHTON L. WELSH: I wish to thank the discussants for their comments. I am glad that they are attempting to treat lupus erythematosus by use of massive amounts of pantothenic acid derivatives and vitamin E.

In regard to Dr. Swartz' comment about vitamin C—I agree with him. Vitamin C enhances the antioxygenic activity of the tocopherols. In general any acid-type inhibitor will promote or energize the activity of any phenolic antitoxidant, such as tocopherol. Furthermore, vitamin C, because of its marked reducing activity, protects vitamin E in the intestinal tract.

Admittedly, the cost of these materials is high. Most of our supplies have been donated, but we have, too, a large number of patients who purchase their own medications. We have encouraged purchase wherever possible, in order to conserve donated materials for patients who cannot afford to purchase. Most patients who have had lupus erythematosus over long intervals, have already had considerable treatment expense, and are not dismayed by one more expensive treatment effort.

It is possible for a local pharmacy to buy bulk calcium pantothenate and vitamin E, calculate, or have the materials capsulated at reasonable prices. Under one such arrangement, the price of calcium pantothenate, 0.5 gm. capsules, and of vitamin E, 100 mg. capsules, is approximately $7.50 per hundred. At that rate, cost of massive-dose therapy is not excessive.

Study of both agents is continuing and is being extended to include the part played by vitamin C in achieving favorable response; the blood levels of pantothenic acid and vitamin E in patients with lupus erythematosus, while "on" and "off" massive-dose therapy, during exacerbations, remissions, and relapses.

Study results are incomplete to date, but results which have been obtained are included in the text of this paper.

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What is the optimal dose of vitamin E in man? Also a contribution to the effect of E-overdosing on the ovary and hypophysis.

by Prof. Dr. Hellmuth Winkler

The synthesis of the vitamins and their purification no doubt provides the possibility to work with experimental accuracy and at the same time to protect from the danger of overdosing. The critical level will be recognized first when damages result from the therapy. Animal tests with overfeeding of vitamins leading to characteristic changes, not with the water-soluble, but indeed with the fat-soluble vitamins are known. These facts are tested and well founded, by means of animal experiments, for vitamins A and D. The requirement of man was estimated at 2-5 mg carotene or 0.1-0.3 mg vitamin A daily. Following administration of larger doses, rats and mice exhibit lose of weight, loss of hair on themuzzle, fatty liver degeneration, hypertrophy of the thyroid gland, and hemorrhag in theintestines and lungs. Exophthalmus and a tendency toward spon taneous fractures are also observed. Large quantities of carotene, according to Sherwood, lead to an increased growth of the vaginal epithelium. A-hypervitaminosis in man is not known.

The daily requirement of vitamin D for man amounts to 2 micro grams for healthy children, the optimal 10 micrograms D_2. The vitamin requirement is not dependent on the vitamin intake alone, but also on the proportion of phosphorus and calcium in thefood. It is increased during puberty, limited to the length of time of growth, during pregnancy and by infectious diseases. High doses lead to pronounced toxic effects in rabbits, rats, mice, guinea pigs, cats and dogs. The bones are decalcified, the calcium level in the blood and urine increased sharply and calcium was deposited in the organs and vessels. The danger of hypervitaminosis in not dependent solely upon the intake, but, reasonably, also on the fate of the respective subatances in the organism and it raises the question of whether a pronounced organ-specificity also exists for the substances.

To the fat-soluble vitamins belongs the anti-sterility factor Already on the basis of its solubility rate there appears here the danger of injury through over-dosing. Since a large part of the excess is excreted via the feces, Guggisberg, Vogt, Bomskov and V. Kaulla as well as John maintain that its therapeutic breadth is very great and repudiate the danger of a hypervitaminosis, Demole has, in abundant tests with tocopherol over 2 months in rats, rabbits, dags, cats, monkeys, and frogs, established the compatibility of doses of 50 mg/kg of body weight without injury. In contrast to this exist the animal experiments and clinical observations of Fecht, Reiss, Roth, and Stahler, which result in completely different outcomes.

Stahler reported earliest that two-thirds of male rats exhibited severe testicular degeneration, as in E-deficiency, after 7-8 weeks of over-feeding with 0.5 mg wheat-germ-oil concentrate. He observed that it is possible that this injury could have resulted from the fat-feeding alone. His tests with synthetic

tocopherol are not yet completed. Also, with female rats, the over-feeding resulted in a sterility. They no longer produced, no mature follicles were found in the ovaries, and the uteri were scarred. Reiss was able to establish that, with synthetic vitamin E as well as with wheat-germ-oil, the periodicity of the estrus cycle was shifted, the estrus cycle became irregular and therefore an ovulation disorder was apparent. The weight of the ovaries decreased just as the number of corpora lutea; fewer follicles matured. In the thyroid, pituitary and uterus neither morphological nor significant weight changes were found. Hereby it was the same whether the substance was administered perorally or parentally. Fecht called the notice to 5 healthy married couples who for months took large quantities of vitamin E without any controls. In the men was found oligospermy and in the women severe disturbances of the estrus cycle, which vanished after discontinuation of the therapy. Roth also reported on similar observations.

I have therefore first endeavored to investigate the optimal dose in man through continuous auditing of the hormone excretion. The pregnandiol excretion was determined as well as the excretion pattern of the progesterone, which can be considered as a reliable test for the corpus luteum function. The determination of the follicle-hormone excretion was not limited only to the free, but also to the bound estrone and estriol in order to also obtain an overview of the biological activity of the individual hormone functions. Likewise the gonadotropic hormones of the HVL* were determined, of which only prolane A could be demonstrated in tne ~~non-pregnant women~~.

The optimal dose resulted from 30 mg per day, which not only was capable of stimulating hormone production but also, as proven by comparable animal tests, caused an increase in the resultant organs, that is the stimulus threshold decreased. This sign was attained in man not only for the corpus luteum during menstruation, but also for the corpus luteum during pregnancy. Here was the increased production of the yellow bodies in the corpus luteum-insufficiency, which was found clinically in threatened abortion, especially clear. They were attained in 4-5 months. They amounted to triple the initial value.

This stimulation has its optimum at 30 mg. Higher doses were capable of increasing the defect by no more. With control of the estrone activity by tocopherol the stimulatory effect, which was measured in a genitalically healthy woman, amounted to over 50%; the potentiating effect in castrated test animals (rats) amounted to 25%. With the first, three reaction types were distinguishable, as follows: a) with an increase of the free estrone, b) with an increase of the bound portion and c) without influence on the excretion (Winkler and Cyrenius).

The prolane excretion shows likewise the dose of 30 mg to be optimal, since it caused the premenstrual excretion to be increased from 6 to 15 ME. From these tests it is concluded that the anti-sterility factor, in addition to its potentiating effect on the castrated rat, produced a genuine stimulating effect on the sexually mature woman, and even therein seemed to present an eminent therapeutic significance for the group of infantile-hypoplastics.

In October 1941 at the 26th Congress of the German Society for Gynecology I was able to report on these results and further

* anterior lobe of the pituitary

to bring to notice that further increasing of the doses did not influence their effect. At that time investigations were presented with a doubled quantity of tocopherol (60 mg). They repudiated the danger of an overdose. Similar observations, reported by Fecht and Roth, were also produced in a local clinic and supported this hypothesis. Especially remarkable was the disturbance of the rhythm of the women who had taken vitamin E for a long time without control; also oligospermy with not only reduced sperm count but also poor motility was found in male ejaculates. These disturbance are interpreted as being caused in the women only by ovarian insufficiency. There remains the question of whether the damage primarily affects the ovary or whether the ovary is influenced through the impaired gonadotropic impulse of the HVL. Corresponding to the stimulating effect through small doses it was assumed that high doses given over a short time or average doses given over a longer time could, according to the Arndt-Schulz'schen rule, overturn their biological effect and, after a temporary increase, result in a paralysis and reduction of the hormone production. Solely quantitative hormone determinations offer the prospect of providing an experimentally reliable groundwork for this hypothesis in man. It seems appropriate to decide the question of whether nervous impulses, the significance of which Stieve first pointed out, also play a decisive role.

Procedure

We gave 300 mg tocopherol per day per os and determined, by continuous auditing of the normal hormone excretion titers, the prolane, estrone and estriol excretion. Tests of the influence of pregnandiol-excretion were performed in phases. In cycles in advance, the normal follicle hormone excretion and the free and bound estrone as well as the free and bound estriol were determined

The follicle hormone excretion (F.H.-excretion) was determine by the modified, by us, Kober'schen Method in a Pulfrich step-photometer, which contained only low limits of error and which tock quick readings on small quantities of animal material. The prolane determination followed after previous concentration of thedaily quantity of urine by the wolframic acid-brucine method according to Katzmann and Doisy in the customary method of the Aschheim-Zondek reaction. It was not only for themacroscopic condition of the uteru and ovaries, but also for the state of the vaginal mucosa, through swabs, outlawed (sic).

As test subjects served healthy, normally menstruating school girls of the Hebammen-Lehranstalt who had already been to the clini for a long time and whose cycles were satisfactorily stabilized. They placed themselves voluntarily at the disposal of this investigation. For 25 days they received 30 mg tocopherol during the cours of a cycle. After 2 months the dose was increased ten-fold, to 300 mg. This pause was intentionally included in order to guarentee the excretion in this time of the smaller quantities given earlier and to exclude any additive effects.

Case M. (Fig. 1): The value for the free estrone in the untreated cycle was found to be highest at the time of menstruation and also increased on the 10th day of the cycle, to be sure only for a short time. At the time of the follicle rupture the expected summit was not reached. The maximum quantity excreted was between 260 and 280 micrograms. On the 13th day of the cycle the administra tion of vitamin E was begun. The first distinct increase was found

on day 21 of the cycle, at which this value was about 390 micrograms, and also premenstrually, where this value was reached or barely exceeded. The increase is the most distinct at the end of the menses, thus at the end of the desquamation and at the beginning of the proliferation, where the value, to be sure, rose to 550 micrograms only for a short time.

The excretion of the bound fraction took a course neither before nor after E-administration parallel to that of the free fraction, which generally in the case of an increase of the bound follicle hormone is not recorded. The initial value was not exceeded. Indeed, the curve had shifted so far that after E the maximum excretion lay around days 22-23 of thecycle. First at the time of the menses and likewise again at the beginning of the regeneration and proliferation phases higher values were reached, and indeed these quantities were around 80 micrograms higher than those of the untreated cycle.

The curve of the total excreted follicle hormone is manifested primarily through the increased excretion of the free fraction, only to a small part through the increase of the bound fraction; through shifting of the peaks a clearly different picture in comparison to the untreated cycle is shown. Comparison of the excretion quantities during the cycles with one another finds the greatest increase around days 19-23, where the excreted quantity steeply increases and exceeds the value for the untreated cycle by about 570 micrograms. The influence was likewise very clear at the end of the desquamation phase and at the beginning of the regeneration phase, where the value likewise increases to about double.

After increasing the doses to 300 mg the excretion curves for total estrone show, just as for the individual fractions, distinct changes, which are the most prominent with the total estrone. Here the initial value lies, as in the untreated cycle, between 200 and 300 micrograms; however only one peak, which lies about 150 micrograms under that of the untreated cycle,was reached after 10 days at the time of menstruation. Also, the maximum during the follicle rupture in only minimally pronounced. Only at the beginning of the following cycle did the excretion of the total estrone increase post-menstrually to the 400 microgram level for a short time, then sank again at once to the previous average value of 200 micrograms. The disturbance of the rythm which occurred with the high-dose E-administration is remarkable. The cycle, which is usually 28-32 days was shortened to 22 days, to be followed by one increased to 32 days.

From the curves of the free estrone and of the bound fraction it is clear that the strong reduction of the total excretion results from the expenditure of both parts. The initial values of the bound estrone, free and bound estriol proceeded at first from thr same height as the normal excretion. At the time of the menses, however, they reached a peak, which was increased only about 50 micrograms above that of the untreated cycle. Thereafter the value fell to under 200 micrograms and slightly increased again only one time at the beginning of the following period. While the excretion value on the cycle treated with 30 mg was already increased more than double after 5 days, after administration of 300 mg one can not speak of an increase at all, rather the values proceeded for the most part below the normal, moreover atypically, that is, without the expected elevated excretion peaks at the time of the follicle rupturing and at the menses.

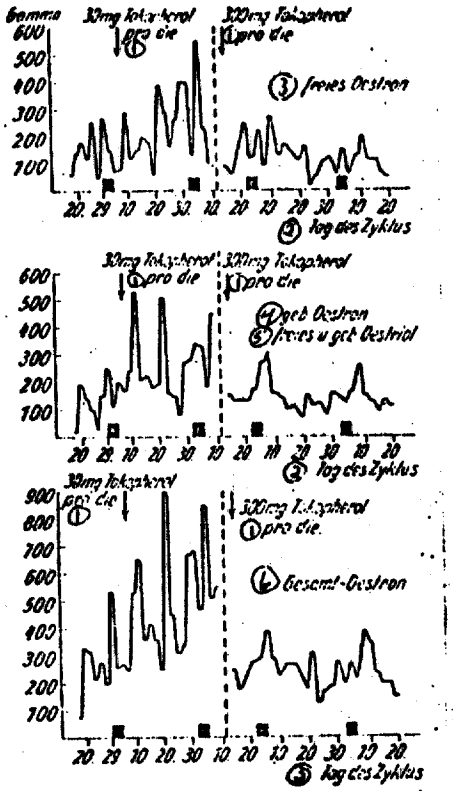

Fig. 1. Case M

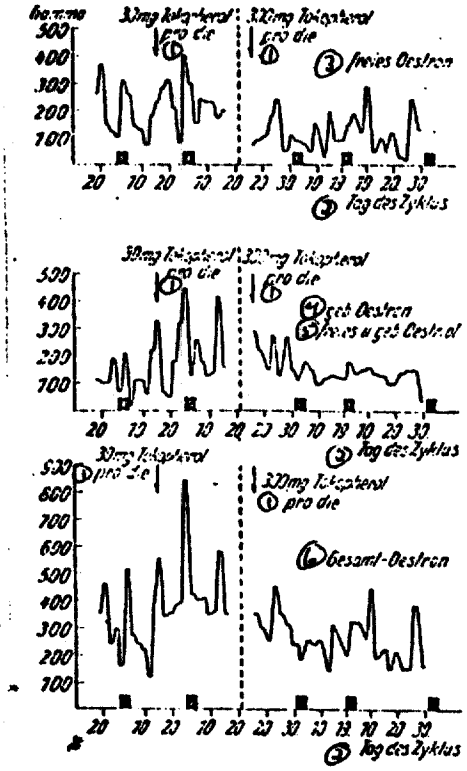

Fig. 2. Case N

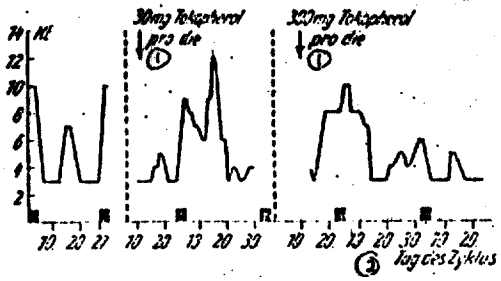

Fig. 3. Case L

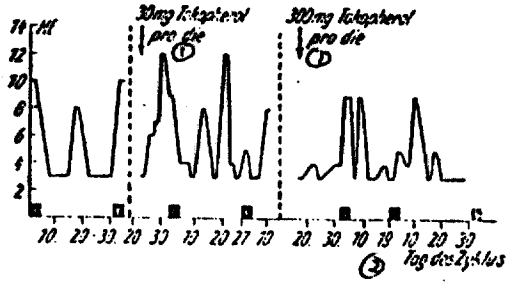

Fig. 4. Case R

Key:
1. per day
2. day of the cycle
3. free estrone
4. bound estrone
5. free and bound estriol
6. total estrone

The same held for the free estrone. Also here after 300 mg, the peak after 15 days, which was especially distinct with the ten-fold lower dose, failed to appear. The value lay at the same height as before the treatment. The menstrual ascent was initially still attained. After 17 days, that is after administration of 5100 mg = 5.1 g tocopherol, the value clearly fell and lay completely below 200 micrograms. Also the increase at the time of menstruation and of follicle rupturing changed no more in regard to that.

With all of the other test persons we could make the same observations on the excretion curves. As especially characteristic, we are inclined to place case N outside. Here also the value of the cycle treated with 300 mg lies, in all but a small exception, below that for the untreated.

Case N. (Fig. 2): The curves show differences from the first, and indeed it is interesting to see that the excretion value for the free estrone of the uninfluenced cycle lies on the average substantially higher than in case M, since here it is raised almost to 400 micrograms while the highest value in the first case amounted to only 280 micrograms. A substantial increase could not be attained through E-administration. The highest value at the time of the menses was only about 90 micrograms above the corresponding value the uninfluenced cycle. All other quantities lay at the same height. In contrast to the free estrone and also in contrast to the remaining fractions in case M, a substantial difference was found here in the excretion of the bound estrone. The maximum at the time of the follicle rupturing (day 15) was only exceeded by around 100 micrograms, the menstrual peak value by about 240 micrograms. These curves allow a distinct difference to be demonstrated through addition of the free and bound estrone and estriol. The maximum at the time of the menstruation is considerably increased except that here the bound, that is the biologically inactive, increased. To what extent this is effective is tested in further investigations.

As a result of the ten-fold increase of the dose the excretion of the total estrone reacted after 10 days with a distinct fall in the curve, which is so great that the value reached no menstrual peaks and lay approximately 250 micrograms below that of the untreated cycle Not till the 15th day of the cycle did it increase again, whereby one can again not speak of the expected maximum, since it exceeded the average value only by about 100 micrograms. All of the excretion quantities of this cycle lay below those of the cycle treated with 30 mg and caused the peaks to be missing.Not till the following cycle is an increase to be observed again, though here also the maximum excretion value for the total estrone was approximately half of that of the cycle treated with 30 mg.

By consideration of the excretion curve of the individual estrone-fractions, in contrast to those of case M, it was possible to determine here that the reduction of the value resulted exclusively from the expenditure of the bound portion. This never exceeded the 200 microgram limit in relation to the values between 200 and 400 micrograms in the cycle treated with 30 mg and allowed both maximums to be missing. The curve of the free estrone is similar in its course to those of the untreated cycle and that treated with 30 mg; the value lies at all times about 100-200 micrograms lower, increasing typically menstrually however.

If the fall of the excretion, especially for the bound fractions in case N, is not clear at first, then we are inclined to make the disturbance of the rhythm, which occurs oppositely here, responsible

for it. In case M the dose of 300 mg replied with a shortening of the cycle to 22 days; it was lengthened in this case to 31 in comparison to the normal 26-28 days.

From these corresponding results it results that the formation of the follicle hormone is reduced and it must be assumed that the mucosal synthesis in connection with the impaired hormonal impulses was insufficient. We are convinced that by further elevation of the doses the values will be yet further reduced, but it is the meaning of this work to show initial injuries and thereby avoid clinical manifestations of them. The critical level lies in our opinion where the changes are especially distinct, at 7 g; if this is given within 23 days, then with this dose the excretion no longer serves to protect the organism from hypervitaminosis.

The disturbances of the rhythn which we have observed are also doubtlessly based upon the hormonal changes. Since a reduction of the corpus luteum activity is also shown by the pregnandiol determination, there can exist no doubt that the mucosal synthesis is insufficient. With lower hormonal impulses a longer time is necessary at proliferation and also the secretory changes will be incomplete. The intensity of this impulse and the formation of the follicle hormone is, as we know, primarily dependent upon the condition of the near-ripe follicles, and it seems possible to us to entirely prevent their ripening by adequately high over-dosing and to make this morphologically comprehensible. For that the animal tests of Reiss also provide evidence which indicates that fewer follicles ripened, that the number of corpora lutea decreased and that the ovarian weight likewise diminished. Stahler has - apparently with yet higher doses - obtained a sterility in female rats, which thereby was conditional in that in the ovaries in general no ripe follicles were found. Since the corpus luteum develops from the granulosa cells of the follicle and thus has the same substratum, changes of a functional manner were also expected here from the first which- as our tests show- are already made distinct at relatively low doses in a reduction of the pregnandiol excretion. Both hormone producers, follicle and yellow body, were affected in the same manner. Clinically it can manifest with the slight reactions, whose curves we have already shown, not only an earlier but also a later menstruation. We see in the repeated menses, according to R. Schroder, a weakness of the mature eggs, which die sooner than normal. The repeated menstruation is certainly the easiest grade of the generative insufficiency, which after abolition of the causative damage can always be balanced, and, so long as they exist, indicate a difficulty in conception.

Through these investigations is the proof produced that high doses of vitamin E lead to a reduction of the F.H.-excretion. The presnt investigations allow the acceptance of the same for the yellow-body function. They find their expression in disturbances of the menses rhythm. Since we also found the same changes in the cycle with women who had received small doses of vitamin E over a long time, the conclusion is permitted that even this disturbance of the cycle is a sign of an ovarian insufficiency. The practical claim which results from this is clear. Even low doses should be allowed to be given for a limited time. Thirty mg tocopherol per day for 6-8 weeks resulted at no time in injury. Thereafter, however, a long pause is necessary. This dosing involved, as I could show in earlier investigations, in fact a stimulation of the hormone production not only in the follicle but also in the yellow-bodies.

The question however, which was already touched upon, where the

primary site of these damages lay, whether in the ovary itself or in the superposed, hormonal center of the hypophysis, remained still open. The reduction, that the injury in the HVL caused, was indeed obvious, because both hormones of the ovaries were quantitatively reduced. They gained in plausibility through the investigation, over which I could report a year earlier, that even the stimulation through low doses proceeds through the HVL.

Multiple relations between vitamin E and the pituitary are known. With an E-deficient diet one finds morphological changes in the pituitary of male animals, with females, these findings are not raised. C. Muller found in the slightly enlarged front lobes numerous, vesicularly distended cells with vacuoles interspersed, which were arranged here and there in small groups. To some ectent the vacuoles flowed together, by which means it approached the formation of typical Schleidt'scher "signet ring" cells. Likewise, vacuole formation was perceived in the eosinophilic cells; eosinophilic "signet ring" cells were also often observed. Geller found these changes in the pituitary even before the deficiency systems appeared in the testicles. Gierhake could, through an increase in vitamin E, cancel this vacuolar degeneration. Stahler attempted to bring to disappearance the "signet ring" cells in castrated rabbits nourished for 2 months with an E-dificiency through administration of 20 micrograms of estradiol benzoate given within 8 days. This was not accomplished. Simultaneous E-administration however allowed a good effect to be realized in the same time. Whether after a long endured sterility a normalization of the pituitary can be reached through the exclusive administration of E is not yet known. Bomskov, Stein and Juhass-Schaffer have, to be sure, in no case seen changes in the pituitary.

We have, therefore, with the same test subjects and the same test procedure, simultaneously determined the prolane excretion and also reproduced here, from a great deal of test material, two typical curves.

Case L. (Fig. 3): Figure 3 gives first of all the prolane-A excretion in the untreated cycle. The maximal value appears during the menses, up to 10 ME. At the time of the follicle rupture it reached only a height of 7 ME. With 30 mg vitamin E at the beginning a fall in the curve is perceived, which is also distinct in the menstrual peak, which is about 1 ME below that of the untreated cycle. Then the value fell post-menstrually again no further than for 3 ME, but reached during the follicle rupturing a height of 12 ME, which was an increase of some 100% in comparison to the normal excretion. Only then did it sink again. The following menstrual maximum could unfortunately no longer be determined.

With the administration of 300 mg the prolane excretion increased here the menses occurred within 16 days, completely normally to 10 ME and maintained this peak for a long time at 8 ME. In the following it fluctuated only between 3 and 5 ME, without exhibiting the previously so distinctly observed maximum at the time of the follicle rupturing. At this time the total administered E-quantity amounted to 8.1 g. On day 16 of the cycle the value lay at 3 ME, which signified a reduction of approximately 50% in comparison to the normal excretion and a reduction of approximately 75% in comparison to the cycle treated with 30 mg. Also, during the menses a fall of about 40% is recorded.

Case R. (Fig. 4): After administration of 30 mg the prolane excretion here exhibits a course which is very similar to that of the untreated cycle, only the value at the time of the menses is 2 ME higher. That indicates an increase of about 20%. On the other hand,

the excretion with 300 mg reacts with a reduction of the menstrual value to 9 ME, which thus lies about 1 ME below the normal excretion and about 3 ME below that of the cycle treated with 30 mg. After that no increase is exhibited on day 16 of the cycle; the cycle is shortened to 19 days and also the following menstrual peak is displaced by about 6 days.

From these two curves it becomes obvious that also in the prolane A excretion a reduction occurs. It becomes especially distinct by an incipient over-dosing at the time of the functional maximum. We see also that this is made especially perceptible by the follicle rupturing, at which time the strongest impulse of the follicle-maturing-hormone is necessary for the rupturing of the follicle. That this must have been a reaction of the ovary is accepted without further ado. It seemed from the first glance amazing and our knowledge of the retarding effect of the follicle hormone on the prolane excretion contradictory that by a reduction of the follicle hormone the prolane excretion, which must increase in this case, likewise sank. And we know that postmenstrually the HVL excreted increased follicle-maturing-hormone, which in the ovary caused maturation of the follicle and consequent elevation of the F.H.-excretion. As soon as the follicle hormone had attained a certain blood level, it impeded the HVL, by which the prolane-A excretion was again reduced. This had then on its part after the follicle rupturing resulted in a reduction of the F.H. formation. Hereby can the HVL again augment the hormone amd indeed now excrete prolane A and B, whereby a second, higher maximum of the F.H.-formation is brought about. This intense excretion retards on its part again the secretion of prolane A and B and by attainment of the maximum it arrives at the degenerative procedure in the corpus luteum, which has as a result the degeneration of the mucosa as menstrual bleeding. The temporal, almost simultaneous reduction of the follicle hormone and prolane A, which becomes especially clear after doses of 7 to 8 g tocopherol, indicates therefore, that the hormonal impulses of the HVL, which as a superposed and sensitive endocrine organ was injured first, no longer sufficed as an adequate F.H.-secretion. These distinctions are naturally made rather perceptible by functional, quantitative hormone analyses, as they are morphologically tangible. Therefore, no doubt can continue that by overdosing, that is by tocopherol doses which nowadays many times still lie within therapeutic limits, injuries to the formation of gonadotropic hormones of the HVL occur, which simultaneously effect the follicle hormone as well as the yellow-body-hormone formation. The assumption that these injuries affect only the gonadtropic hormones and should spare the other adrenotropic and metabolic hormones is construed. We maintain that injuries are also possible here. It can therefore urgently be warned against excessively long and high doses of vitamin E.

Discussion of the results

Quantitative investigations of the F.H.-, prolane-, and corpus luteum hormone-excretion show that in contrast to the uninfluenced cycle with low doses of tocopherol an increased production is possible in the sense of a genuine increase in hormone formation. With elevation of the doses ten-fold, 300 mg per day, there occurred after 10 days a reduction in production, which manifested itself in a clear reduction of hormone excretion and uniformly reduced the excretion of F.H. as well as corpus luteum hormone and prolane. The

reduction in production was especially evident by all three factors at the time of the functional maximum, that is at the time of the menses and of the follicle rupture. The critical level lay at 7-8 g with a daily administration of 300 mg. At this dose the excretion no longer sufficed to protect the organism from hypervitaminosis.

The damage affects primarily the HVL and works through the influence of the gonadotropic hormone of the HVL on F.H.- and yellow body-hormone-excretion with the same intensity. It seems questionable whether the formation of the gonadotropic hormones alone is impaired. In spite of missing morphological findings, it is assumed that this damage is also for the other adenotropic and metabolic hormones.

This hypervitaminosis, which goes hand in hand with a reduction in the production of the sexual hormone, causes clinical disturbances of the rhythm, which can appear as proiomenorrhea as well as opsomenorrhea. These disturbances of rhythm are the expression of an ovarian- and as we can say on the basis of this investigation- and a pituitary insufficiency.

The practical claim of it is that even low doses of vitamin E should be given only for a limited period. 30 mg per day for 6-8 weeks led at no time to injury. Thereupon, however, a long pause is necessary, since here lies the therapeutic limit.

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HOFFMANN-LA ROCHE INC.

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March 8, 1974

Bureau of Foods
Food and Drug Administration
GRAS Review Branch (BF-335)
200 C Street, S. W.
Washington, D. C. 20204

Gentlemen:

Re: Tocopherols -- GRAS Review

Please refer to our submission dated November 26, 1973. Attached please find an amendment to that submission providing an additional report.

Under separate cover, ten copies of this information are being supplied to:

Select Committee on GRAS Substances
Federation of American Societies for
Experimental Biology
9650 Rockville Pike
Bethesda, Maryland 20014

The original and two copies are enclosed herein.

Sincerely,

HOFFMANN-LA ROCHE INC.

Raymond E. McKinley, V.M.D.
Assistant Director
Drug Regulatory Affairs

REM:kr
Attachment
NDA No. 74291
cc: Select Committee on GRAS Substances (10)

January 18, 1974 Dr. R. M. Salkeld

High-Dose Vitamin E Therapy

Summary

Part of the literature on high-dose vitamin E therapy is reviewed in an interim report. Doses of 3,000 IU for up to 13 weeks and 2,000 IU for up to 3 years have no detrimental effect on a variety of clinical and biochemical parameters. These high doses are well tolerated; only 1 to 2% of 2,500 patients complained of some side effect -- mostly gastrointestinal disturbance. Intermittent claudication is the only indication for which vitamin E is of therapeutic value.

R. M. Salkeld

1. Introduction

This interim report on vitamin E derives from a study of the first part of the dossier which is being assembled and which will eventually cover all aspects of high dose ($\geqslant$ 200 IU* daily) vitamin E therapy in humans - indications, contra-indications, side-effects, effective dose, absorption, effect on clinical and biochemical parameters.

2. Indications

Vitamin E has been recommended in the treatment of a multitude of conditions but only for a few of them is there convincing evidence derived from double-blind controlled trials on an adequate number of patients.

Aging - The 10 year survival rate of 1476 patients with intermittent claudication and probably generalized atherosclerosis treated with 400 IU synthetic α-tocopherol acetate daily was higher than in any of 9 other series treated without vitamin E (8).

Angina pectoris - No unequivocal improvement was found with 300 IU daily for up to 24 weeks in two trials one of which was a double-blind matched pairs study (4, 37).

Arterial disease - Gangrene and Raynaud's disease - excellent results are reported in 8 cases (5, 44).

Athletic performance - In a double-blind controlled trial with 13 matched pairs of schoolboys 13 - 18 years old, 400 IU daily for 6 weeks had no effect on athletic performance (42).

Chronic cystic mastitis - 200 - 400 IU daily for 3 - 4 months gave complete symptomatic relief in 80 % and clinical improvement in 65 % of 36 cases (1, 39).

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* 1 mg d,l-α-tocopherol acetate = 1 IU
1 mg d-α-tocopherol acetate = 1.36 IU
1 mg d,l-α-tocopherol = 0.68 IU
1 mg d-α-tocopherol = 0.92 IU

Congestive cardiac failure - No effect reported in 11 cases on 300 IU daily for mostly $\leq$ 3 months (4).

Diabetes mellitus - In one trial (49) 40 of 90 diabetics could reduce their insulin dosage by an average of 13 units (vitamin E dose not given), whereas in another no improvement was noticed in 27 patients on 400 IU daily for up to 13 weeks (34).

Digitalis intoxication - In 30 patients with 35 incidents of digitalis intoxication, vitamin E was always successful in treatment irrespective of the cardiac glucoside involved. A dose of 200 - 500 IU daily orally (onset 12 - 24 hours) or intravenous (onset 1 - 6 hours) was used (19, 20). Vitamin E was used prophylactically to allow continued digitalis therapy.

Dupuytren's contracture - Very slight improvement (reduction of contracture angle) was seen in 15 of 26 hands treated with 300 IU daily for 43 weeks (25). In contrast, trials using 200 IU (36) or lasting only up to 8 weeks (24) showed no positive results.

Gastric ulcer - 100 - 200 IU daily intramuscularly for one month followed by 40 IU orally were reported to produce rapid improvement in all of over 100 cases and complete remission in many (11).

Gravitational ulcers - Good results were obtained in 3 trials of vitamin E, 250 - 375 IU daily for up to 10 weeks (no controls). In 15 patients there was an impression of quicker healing than achieved by local treatment or compression bandage (41); of 25 patients 88 % had excellent or good results (5) and in 13 cases success was reported in 100 % (44).

Hypercholesterolaemia - 40 g (sic) d-α-tocopherol acetate (54,400 IU) daily for 4 weeks depressed the serum cholesterol by 100 mg/100 ml in 2 of 3 hypercholesterolaemic patients, with rebound to pretreatment levels on discontinuing vitamin E (16).

Hypertension - No effect on blood pressure was found in 5 cases of mild hypertension administered 300 IU daily for up to 30 weeks (4).

Intermittent claudication - Negative findings stem from studies which were too short (18) or used inadequate doses. There are many papers, including double-blind controlled trials, which show that $\geq$ 400 IU daily for $\geq$ 3 months improves the walking distance considerably in grade II (Boyd) patients (8, 17, 27, 29, 35, 56). In patients fit enough to undergo operation, lumbar ganglionectomy gives slightly better results (7).

Lupus erythematosus - In all of 32 cases treated with 50 - 660 IU daily for 2 - 36 months there was 70 - 100 % clearing of skin lesions (53). In a series confirmed by biopsy and treated with 1,000 - 2,000 IU daily 90 - 100 % clearing was achieved in over 75 % of cases (54). However, 10 patients receiving 360 - 500 IU daily for 14 weeks showed no improvement (23).

Menopause - Of 48 cases with severe symptoms (sweats, flushing), 18 were administered placebo for up to 6 weeks and one was cured. The remaining 47 were then treated with up to 1,000 IU daily for up to 14 weeks. 65 % were completely or markedly improved and of these 70 % relapsed on discontinuing vitamin E, suggesting that the recovery was not spontaneous (30).
In all of 10 cases of menopause pruritus (no controls) 300 IU daily produced excellent or good results (5).

Muscle cramps - In 126 cases of leg cramps (no controls) treated with 300 - 400 IU daily there was 100 % success (2, 10). In 242 cases of back muscle cramps (no controls) 400 IU daily for at least 6 - 8 weeks achieved good or moderate results in 58 % of acute cases and 93 % of chronic cases (22).

Porphyria - In 4 patients with porphyria clinical and biochemical improvement was achieved by administration of 100 IU daily for 4 weeks (32); the type of porphyria is mentioned for only 2 of the cases, and even these diagnoses are in doubt (52). No effect of vitamin E therapy was seen in two further series: 5 cases treated with 100 - 200 IU daily for 4 weeks (31) and 4 cases (no dose or duration mentioned) (52).

Thrombocytopenic purpura - 200 - 400 IU daily were completely successful in 5 cases, the platelet count and capillary fragility returning to normal within 7 - 14 days (45).

Thyrotoxicosis - In 3 cases of severe thyrotoxicosis treatment with 200 IU daily for 1 - 2 months returned basal metabolic rate, exophthalmos and symptoms of nervousness towards normal (11).

Venous thrombosis - In 166 cases of chronic phlebitis 300 - 2,000 IU daily (duration not mentioned) achieved good or complete relief of symptoms in 67 %. Best results were produced by a dose $\geq$ 800 IU daily (43).

In 69 cases of acute and subacute thrombophlebitis 500 and 550 IU daily respectively achieved complete cure in 100 % of the 24 acute cases within 3 weeks, and good or fair results in 98 % of the 45 subacute cases within 3 months. No other treatment was administered. There were no controls (48).

A regimen of 200 IU daily orally and 10 ml intravenous 10 % calcium gluconate on alternate days was used prophylactically against post-operative thrombo-embolism. In 100 cases a significant reduction in comparison with the 100 control patients was achieved in the incidence of thrombosis - 10 mild, 0 severe (control: 3 mild, 10 severe) and pulmonary embolism - 0 (control: 2) (57).

3. Side-effects

In table 1 the incidence of side-effects at various daily dosages and durations is summarized. In trials involving 1,910 patients side-effects were not observed (or did not merit recording). In 6 trials involving 466 patients about 7 - 9 % complained of some side-effect - mostly gastrointestinal disturbance.

Table 2 lists other individual cases of side-effects reported to be due to vitamin E.

4. Clinical and biochemical parameters

Table 3 summarizes the results of investigations into the effect of various dosages of vitamin E and durations of treatment on certain clinical and biochemical parameters. Thiamine levels in blood and urine were reported to be reduced in an Italian study on 19 cases, and the serum cholesterol was lowered in two hypercholesterolaemic patients; also a transitory creatinuria was observed in one case. Otherwise, no clinically significant changes were recorded.

5. Conclusions

From the reported work studied so far, it can be concluded that vitamin E in doses of up to 3,000 IU daily for up to 13 weeks has no detrimental effect on a variety of clinical and biochemical parameters. Also doses of 2,000 IU for 3 years are reported not to cause haematological or urinary abnormalities.

These high doses are well tolerated by the majority of patients. Of about 2,500 cases only 1 - 2 % complained of some side-effect - mostly gastrointestinal disturbance. Even this symptom can be partly prevented by administering the vitamin E in enteric-coated form.

In the literature studied so far, intermittent claudication is the only indication in which vitamin E has been shown to be of value by means of double-blind controlled trials on an adequate number of patients.

The review of the literature continues and will be reported in a second RCR.

Table 1: Incidence of side-effects

Daily dose (IU)	Duration (weeks)	Number of cases		
		No side-effects	Side-effects not mentioned	Side-effects in some patients
200 - 800	4 - 30	151 (14,30, 36,37, 39,48)	507 (1,4,5,8,10, 11,18,19,20, 22,26,31,35, 40,42,44,45, 57,33)	10 - (Lupus erythematosus) a few complained of lassitude, loss of libido, and joint pains (probably due to disease not vitamin E) (23) 13 - 4 complained of headache, nausea, fatigue (24) 27 - 1 (diabetic without insulin, 19 years) needed insulin; many had gastrointestinal disturbances (34) 15 - a few complained of giddiness and palpitations (41)
	30 - 100	20 (3,25)	38 (27)	-
	8 - 150 104 - 260 260 520	19 (53)	461, 607 (8)	400 - 3.5 % had gastrointestinal symptoms (17)
1,000 - 2,000	8 6 - 100 50 - 150 26 30	2 (46) 5 (55) 64 (54)	1 (28) 12 (13)	
2,200	3 - 12 13 - 24 25 - 36 >37		6 3 5 2 (56)	
2,000 ↓ 4,000	5 8			1 - cheilosis, angular stomatitis, gastrointestinal disturbances (21)
2,000 ↓ 00 ↓ 5,000 ↓ 10,000	1 2 3 7		1 (50)	
54,400 (40 g d-α-toc.acetate)	4		6 (16)	

Table 2: Side-effects in isolated cases

Number of cases	Side-effects	Ref.No.
1	Allergic eczematous contact dermatitis due to Tashan Cream (α-tocopherol responsible not other components).	9
1	Generalized dermatitis and pruritus after 16 weeks and 36 g EPHYNAL.	30
Several	Pruritus and pustules after long treatment, 600 mg dl-α-tocopherol i.m.	33
-	Digitalis intoxication said to occur unless digitalis dose reduced by 50 %.	51
1	Severe epigastric pain and vomiting for ½ hour 30 minutes after taking 2 tablets synthetic α-tocopherol acetate on 3 occasions in patient with quiescent duodenal ulcer.	47
-	Atrial fibrillation and supraventricular tachycardia is said to occur in hyperthyroid hearts treated with α-tocopherol	51
-	Hypoglycaemia is said to occur in diabetics unless insulin dose reduced.	49
-	Some cases of oligospermia, azoospermia and loss of potency (in cases of induratio penis plastica) have been observed.	15

Table 3: Effect on various parameters

Parameter	Daily dose (IU)	Duration (weeks)	No. of cases	Findings
Haematology	200 - 660	8 - 150	19	no abnormality (53)
	1,000 - 2,000	50 - 150	64	no abnormality (54)
Serum cholesterol	54,400	4	6	decreased by up to 100 mg/100 ml (16)
	3,000	13	1	no change (21)
	75	8	8	10 % increase (38)
	300	1	23	no change (6, 26)
Urinary creatine	3,000	13	1	transitory creatinuria (21)
ECG	300	15	22	(cardiovascular disease); no change (4)
	3,000	13	1	no change (21)
Blood fatty acids	75	8	8	25 % increase after 1 week (38 5 % increase after 8 weeks
Blood pressure	300	15	41	(cardiovascular disease); no change (4, 37)
	200 - 660	8 - 150	19	no change (53)
Urinary 17-ketosteroids	3,000	13	1	no change (21)
	300	1	20	no change (6)
Urinalysis	200 - 660	8 - 150	19	no abnormality (53)
	1,000 - 2,000	50 - 150	64	no abnormality (54)
Blood and urine thiamine	150 - 600	1	19	35 - 85 % reduction (12)
Blood vitamin A and carotene	3,000	13	1	no change (21)
Liver function tests, blood coagulation, muscle biopsy, exercise tolerance	3,000	13	1	no change (21)

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