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ASCORBIC ACID
& SALTS

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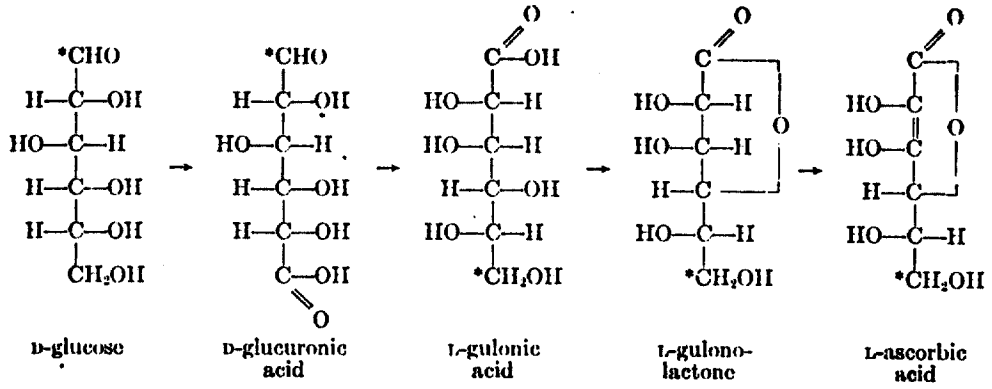


* Denotes the fate of C-1 of D-glucose in this scheme.

Fig. 2. Pathway for the biosynthesis of L-ascorbic acid in the rat.

C-1. The scheme in Figure 2 is in accord with these data since C-1 of glucose would become C-6 of L-ascorbic acid as indicated by the asterisks. Other evidence has also been presented for the biosynthetic pathway in Figure 2. Isherwood, Chen, and Mapson (36) reported that administration of D-glucuronolactone and L-gulonolactone to rats produced an increase in the urinary excretion of L-ascorbic acid. Tracer experiments further established the conversion of D-glucuronolactone and L-gulonolactone to L-ascorbic acid in rats.

Lack of Synthesis in Man, Monkey, and the Guinea Pig. Man, monkey, and the guinea pig are the only mammals known to be unable to synthesize L-ascorbic acid; and thus they require vitamin C in their diet for the prevention of scurvy. (37). An explanation for this defect has come from finding that these species are unable to convert L-gulonolactone to L-ascorbic acid (Fig. 2). Man, monkey, and the guinea pig lack an enzyme in their liver microsomes required for carrying out this important reaction.

Biosynthesis in Plants. Plants appear to form L-ascorbic acid from D-glucose by a pathway other than that given for the rat in Figure 2. Isotopic studies carried out in the ripening strawberry and the germinating cress seedling showed that the vitamin is synthesized from D-glucose by a fairly direct route. In this case D-glucose-1-^{14}C is converted to L-ascorbic acid labeled predominantly in C-1, and D-glucose-6-^{14}C is converted to L-ascorbic acid labeled predominantly in C-6. This differs from the data obtained in the rat where C-1 of glucose yielded C-6 of L-ascorbic acid and C-6 of D-glucose yielded C-1 of L-ascorbic acid. Based on his studies Loewus (38) has postulated that the reactions of the hexose monophosphate shunt may be required for the synthesis of the vitamin in plants. It should be noted that Isherwood and coworkers (36) had demonstrated earlier that L-gulonolactone and D-glucuronolactone are converted to L-ascorbic acid in cress seedlings. Thus, reactions of the animal biosynthesis pathway must also occur to a certain extent in plants.

Glucuronic Acid Pathway. In recent years a new route of glucose metabolism, the glucuronic acid pathway (37), has been demonstrated largely on the basis of studies of the biosynthesis of L-ascorbic acid (Fig. 3). According to this scheme L-gulonic acid serves as a precursor for L-xylulose as well as for L-ascorbic acid. L-Xylulose is the sugar excreted by humans with the metabolic disease, essential pentosuria.

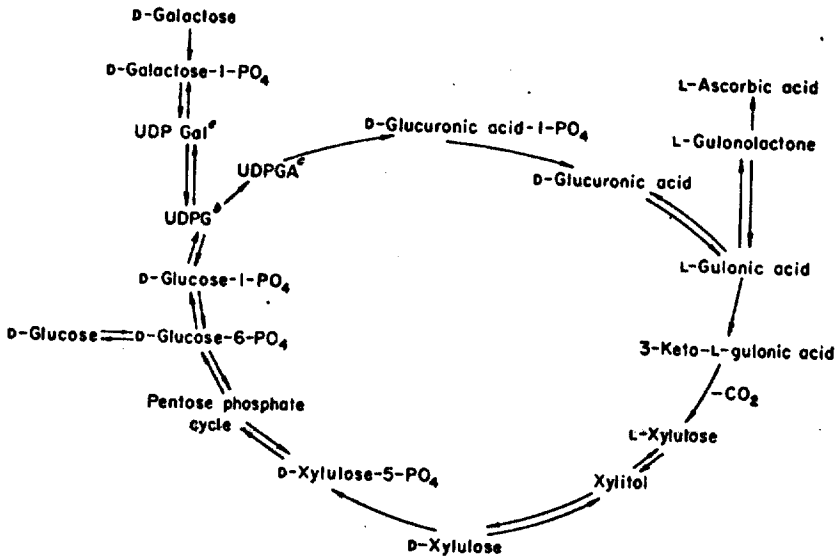


Fig. 3. Glucuronic acid pathway. [a] Uridine diphosphate galactose; [b] uridine diphosphate glucose; [c] uridine diphosphate glucose glucuronic acid.

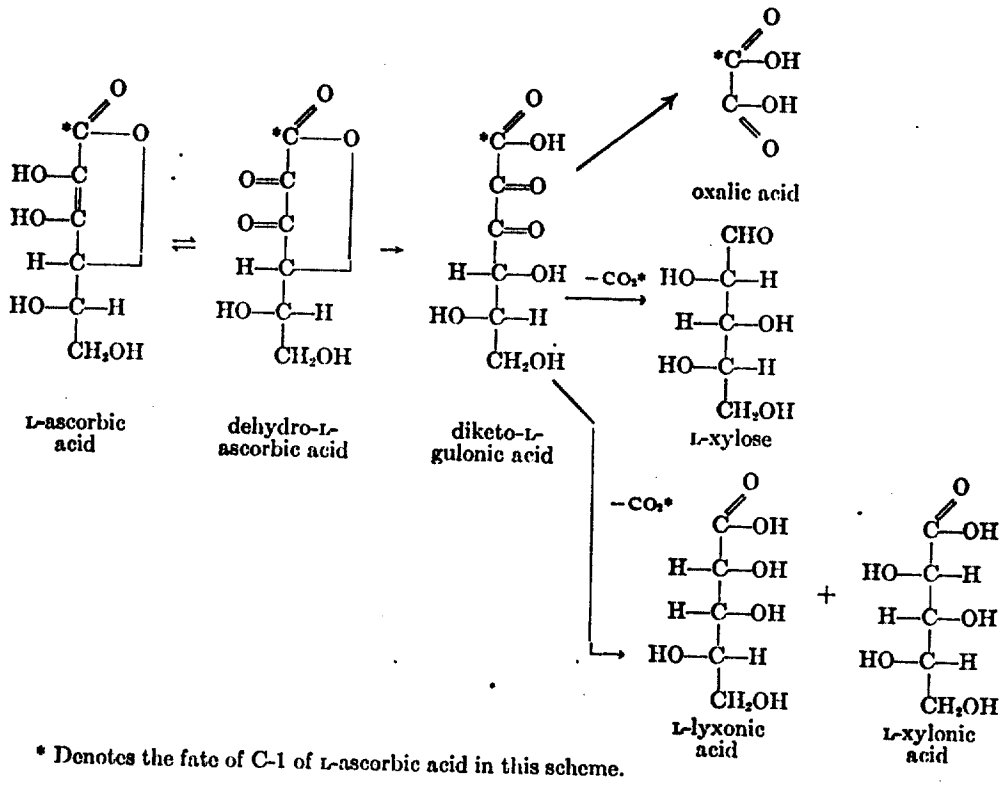
It is of considerable interest that administration of various drugs and other chemicals to rats markedly stimulates the synthesis of L-ascorbic acid via the glucuronic acid pathway (39). Included among these are the hypnotic drugs, chlorobutanol and barbital; the analgesics, aminopyrine and antipyrine; and the carcinogenic hydrocarbons, 3-methylcholanthrene and 3,4-benzpyrene. Two of the drugs, aminopyrine and antipyrine, also increase the urinary excretion of L-xylulose in patients with essential pentosuria.

The mechanism by which these drugs increase the biosynthesis of L-ascorbic acid is not known. It is possible that this effect of drugs on glucose metabolism may represent a new adaptive response on the part of the body to foreign compounds. This is suggested from the observation that those compounds which are potent in stimulating the synthesis of L-ascorbic acid such as barbital, chlorobutanol, and 3-methylcholanthrene, also increase the activity of liver microsomal enzymes which metabolize drugs.

Fate of L-Ascorbic Acid in Animals. Studies with L-ascorbic acid labeled in various positions with ^{14}C have shown that the vitamin is extensively oxidized to respiratory CO_2 in rats and guinea pigs (33). In contrast, no conversion of L-ascorbic acid-1-^{14}C to respiratory CO_2 occurs in man. An injected dose of L-ascorbic acid-1-^{14}C disappears slowly in man with a biological half-life of about 17 days which compares to about 3 days for guinea pigs. These findings are correlated with the much longer time it takes humans to show signs of scurvy. For instance, man requires about 3 months on a vitamin C-free diet to develop scurvy; while the guinea pig becomes markedly scorbutic on such a diet in about 3 weeks.

The first step in the metabolism of L-ascorbic acid is its oxidation to dehydroascorbic acid followed by the formation of 2,3-diketogulonic acid (Fig. 4). A portion of the 2,3-diketogulonic acid thus formed is converted to oxalate which is excreted in urine. 2,3-Diketogulonic acid is decarboxylated in liver to form L-xylose and in kidney to form L-lyxonic and L-xylonic acids.

Distribution of Ascorbic Acid in Animals. L-Ascorbic acid has a unique pattern of distribution in mammalian tissues (32). Several tissues have ascorbic acid concentrations several hundred times that of the plasma, ie, hypophysis, adrenal, thymus, corpus luteum, and retina. A large number of tissues have concentrations 50 to 100 times the plasma level; these include the brain, testicle, thyroid, small intestinal mucosa, lymph glands, lungs, liver, spleen, white blood cells, pancreas, and salivary glands. A number of other tissues have only about 10 times the plasma concentration, ie, kidney; skeletal, smooth, and cardiac muscle; and erythrocytes.



* Denotes the fate of C-1 of L-ascorbic acid in this scheme.

Fig. 4. Fate of L-ascorbic acid in animals.

There is some evidence, confirmed by both chemical and radioactive tracer methods, that ascorbic acid exists in a bound form in guinea pig liver. The significance of this bound ascorbic acid is not known, but the existence of the vitamin as such may serve to protect it against degradation by liver enzymes.

Action of L-Ascorbic Acid on Biochemical Systems

L-Ascorbic acid has been reported to act on many enzyme systems, but definitive information on the specific mechanisms involved is lacking. L-Ascorbic acid does not appear to function as a cofactor in the manner described for the other vitamins, such as biotin, thiamine, and nicotinamide. Knox and Goswami (32) have recently reviewed in detail the various biochemical systems suspected to involve L-ascorbic acid, and some of the more important ones are mentioned.

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The Pharmacological Evaluation of Antioxidants

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I. Introduction

Investigations into the problem of rancidity and methods for stabilizing edible oils and fats have been quite extensive. One of the fundamental problems yet to be solved is the mechanism by which fats become rancid and the role of antioxidants in the prevention or delay of rancidification. Mattill (1947) has reviewed the chemical aspects of the problem. The influence of lipoxidases, yeasts, molds, or bacteria on the decomposition of glycerides of fatty acids has also not been clearly elucidated although it is known that the presence of such entities is not necessary for the production of rancidity.

The importance of preserving the wholesomeness of edible fats is exemplified by the fact that in the United States the average individual derives about one-third of his total caloric requirements from fat. Fat not only functions as a concentrated foodstuff, but also has an important relationship to the availability and utilization of essential dietary substances. Some of these substances are vitamin A, carotene, vitamin E, vitamin D, pantothenic acid, pyridoxine, biotin, ascorbic acid, and linoleic acid (Burr and Barnes, 1943). Considerable experimental evidence is available also which emphasizes that the diet of man and animals is enhanced in some way by the addition of fresh natural edible fats. The presence of rancid fats in the diet, however, has a serious deleterious

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effect, which goes beyond the destruction of essential dietary constituents. Fitzhugh *et al.* (1944) have demonstrated that the feeding of rancid lard to rats at a dietary level of 6% resulted in a marked emaciation of the animals by the end of the first year on the diet. A progressive type of paralysis was also noted in these animals at the time of termination of the experiments (2 years). The pathological examination verified the fact that the major symptoms were the result of a vitamin E deficiency, although the byproducts of the oxidized fat appear to have a toxic action of their own (Burr and Barnes, 1943).

From these considerations the indications are that considerable emphasis must be placed on the importance of keeping edible fats palatable and wholesome, especially when storage is necessary. Stabilization of fats has been accomplished by several means, such as choice of raw materials, hydrogenation, proper packaging, deodorization, and exercising every precaution during processing to avoid unnecessary exposure to heat and light, or contamination with prooxidants. All these methods have their limitations in that their applications are not always feasible to all fats and fatty food products; hence reliance for the stabilization of fats has been placed on antioxidants. By the addition of traces of certain chemicals it has been found that oxidative rancidity can be inhibited and the age resistance of fats considerably increased. This phenomenon has been known for more than 100 years and the first utilization for prevention of rancidity was in the pharmaceutical field—employment of gum benzoin to protect lard. This product has been official in the United States Pharmacopoeia since 1880.

Many compounds have been found to possess antioxidant properties, the more common compounds being gum guaiac, gallic acid, catechol, and hydroquinone. Numerous other chemicals of the phenolic and acidic type have also had their proponents. Naturally occurring antioxidants have received considerable attention, the purpose being to develop a nontoxic substance suitable for food use. Lecithin, cephalin, tocopherols, and products obtained from cereals, yeasts, sugars, and other food substances have been proposed. Lundberg (1947) has compiled a list of antioxidants which are in current use or which have been proposed for use in stabilizing edible fats.

The criteria which are considered necessary for establishing the usefulness of a chemical as an antioxidant have been listed by Higgins and Black (1944). According to these authors, the antioxidant should be soluble in fats and impart no foreign color, odor, or flavor to the fat even on long storage and should be an effective inhibitor in the treated product for at least a year after storage at a temperature of 75°–85° F. in unsealed containers. The antioxidant should be unchanged when

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heated and should possess the ability of retarding rancidity in baked foods prepared from fats treated with the antioxidant. The safety of the antioxidant for food use must be established. There are many antioxidants which are able to pass the physical and chemical criteria for practical usefulness, but to establish proof of nontoxicity is more difficult.

Antioxidants are chemical preservatives and come under the terms of the Federal Food, Drug and Cosmetic Act when they are present in food shipped in interstate commerce. Any antioxidant which may be legally incorporated into foods must be declared on the label in a way to show it to be a preservative. If the chemical is safe and nonpoisonous, it can be incorporated into unstandardized foods as a preservative if it does not conceal damage or inferiority or make the product appear better or of greater value. It may be used in standardized food only if the standard recognizes the preservative as a legitimate ingredient. If the food is not a standardized item and the antioxidant is nonpoisonous, the only requirement is the declaration of the preservative on the label. If the antioxidant is a poisonous and deleterious substance it must be shown that the preservative is required and cannot be avoided in good manufacturing practice. In this event, the law provides that the Administration shall promulgate regulations limiting the quantity of the substance a food may contain so that it will not constitute a hazard to the public health. It is the manufacturer's and shipper's responsibility to secure adequate evidence of safety for the antioxidant that he intends to use. A general plan for the pharmacological investigation, which permits a thorough appraisal of the toxicology of an antioxidant, has been presented (Lehman, 1948).

Of the many chemicals proposed for antioxidant use and under active investigation, pharmacological data on the compounds listed below have become available.

1. Nordihydroguaiaretic acid
2. Propyl gallate
3. Hydroquinone
4. l-ascorbyl palmitate
5. *d*-isoascorbyl palmitate
6. *d*-isoascorbic acid
7. Thiodipropionic acid
8. Dilauryl thiodipropionate
9. Distearyl thiodipropionate
10. Gum guaiac

The palmitates and *d*-isoascorbic acid are not considered very effective as antioxidants and any discussion of their pharmacology will be incidental only. Phenol and catechol are included for the purpose of comparison, although they are too toxic for food use.

II. Acute Toxicity

A summary of the acute toxicity of the antioxidants is presented in Table I. From a practical standpoint it may be stated that an antioxidant which possesses an LD_{50} (the quantity of the antioxidant which is fatal to 50% of the test animals following oral administration) of 1,000 mg./kg. or above has met at least one important criterion of harmlessness. An inspection of the table reveals that nordihydroguaiaretic acid, propyl gallate, thiodipropionic acid and its derivatives, *d*-isoascorbyl palmitate, gum guaiac, and butylated hydroxy anisole have measured up to this criterion. It is of interest to note that in general hydroquinone and phenol have the same order of toxicity.

Table I

The Acute Toxicity of Antioxidants

Animal	Route of administration	Approximate LD_{50} mg./kg.	Source
		Nordihydroguaiaretic acid	
Mice	Oral	>2000	Bieter (1949)
Mice	Oral	4000	F & D A* (1950)
Rats	Oral	>2000	Bieter (1949)
Rats	Oral	5500	F & D A* (1950)
Guinea pigs	Oral	830	Bieter (1949)
Mice	Intraperitoneal	550	Bieter (1949)
		Propyl gallate	
Mice	Oral	2000	Boehm and Williams (1943)
Mice	Oral	3500	F & D A* (1950)
Rats	Oral	3800	Orten *et al.* (1948)
Rats	Oral	3600	F & D A* (1950)
Rats	Intraperitoneal	380	Orten *et al.* (1948)
		Thiodipropionic acid	
Mice	Oral	>2000	Hazleton (1949)
Mice	Oral	2000	F & D A* (1950)
Mice	Intraperitoneal	250	Hazleton (1949)
Mice	Intravenous	175	Hazleton (1949)
Rats	Oral	>2000	Hazleton (1949)
Rats	Oral	3000	F & D A* (1950)
Rats	Intraperitoneal	500	Hazleton (1949)
Rats	Intravenous	>300	Hazleton (1949)
		Dilauryl thiodipropionate	
Mice	Oral	>2000	Hazleton (1949)
Rats	Oral	>2500	Hazleton (1949)
Mice	Intraperitoneal	>2000	Hazleton (1949)

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TABLE I (*Continued*)

Animal	Route of administration	Approximate LD_{50} mg./kg.	Source
		Distearyl thiodipropionate	
Mice	Oral	>2000	Hazleton (1949)
Rats	Oral	>2500	Hazleton (1949)
Mice	Intraperitoneal	>2000	Hazleton (1949)
		d-Isoascorbyl palmitate	
Mice	Oral	>8000	F & D A* (1950)
Rats	Oral	6000	F & D A* (1950)
		Gum guaiac	
Mice	Oral	>2000	Bieter (1949)
Rats	Oral	>2000	Bieter (1949)
Rats	Oral	>5000	F & D A* (1950)
Guinea pigs	Oral	1120	Bieter (1949)
Mice	Intraperitoneal	>2000	Bieter (1949)
		Catechol	
Mice	Oral	260	Bieter (1949)
Rats	Oral	260	Bieter (1949)
Guinea pigs	Oral	210	Bieter (1949)
Mice	Intraperitoneal	190	Bieter (1949)
		Butylated hydroxy anisole	
Mice	Oral	2000	F & D A* (1950)
Rats	Oral	2200	F & D A* (1950)
		Hydroquinone	
Mice	Oral	400	F & D A* (1950)
Rats	Oral	320	F & D A* (1950)
Guinea pigs	Oral	550	F & D A* (1950)
Pigeons	Oral	300	F & D A* (1950)
Dogs	Oral	200	F & D A* (1950)
Cats	Oral	70	F & D A* (1950)
Rats	Intravenous	115	F & D A* (1950)
		Phenol	
Mice	Oral	520	Bieter (1949)
Mice	Oral	395	F & D A* (1950)
Rats	Oral	440	Bieter (1949)
Rats	Oral	450	F & D A* (1950)
Mice	Intraperitoneal	360	Bieter (1949)

* Food and Drug Administration, unpublished data.

III. Chronic Toxicity

1. *Rats*

Lifetime feeding studies in rats have been carried out with all the compounds listed. One of the criteria for establishing the harmlessness of a substance which may be ingested chronically when added to food is the effect of such a substance on the growth rate of rats. The first six

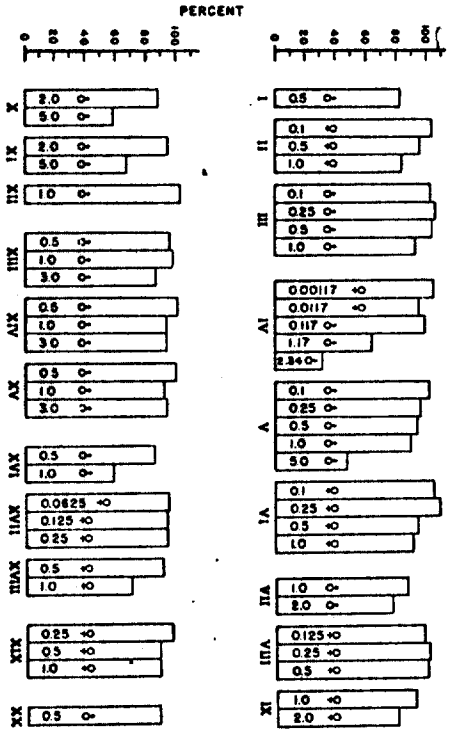

Fig. 1. Mean growth rate of rats fed the antioxidants for 6 months expressed as per cent of the control groups. The growth rate of the control animals was taken as 100 per cent. The figures in each column represent the per cent of the antioxidant in the diet. The symbol ♂ = male; ♀ = female. The Roman numerals identify the antioxidants and source of the data as follows: I, II, Nordihydroguaiaretic acid (Bieter); III, Nordihydroguaiaretic acid (F&DA*); IV, Propyl gallate, (Orten *et al.*); V and VI, Propyl gallate, (F&DA*); VII, VIII, IX, Hydroquinone, (F&DA*); X, *l*-ascorbyl palmitate, (F&DA*); XI, *d*-isoascorbyl palmitate, (F&DA*); XII, *d*-isoascorbic acid, (F&DA*); XIII, Thiodipropionic acid, (Hazleton); XIV, Dilauryl thiodipropionate, (Hazleton); XV, Distearyl thiodipropionate, (Hazleton); XVI, XVII, XVIII, Catechol, (F&DA*); XIX, Phenol, (F&DA*); XX, Gum guaiac, (Bieter).

* Food and Drug Administration, unpublished data.

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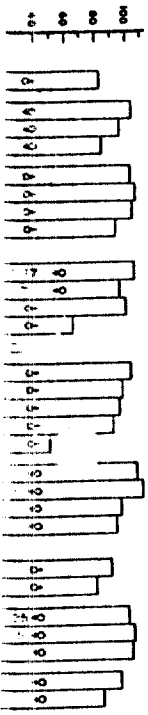

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months in the life of a rat is usually taken for observing growth changes for the reason that this time period represents the rapidly growing stage. Figure 1 represents a summary of the data of the various compounds. Practically all of the antioxidants are recommended for use in quantities not to exceed 0.01% in animal fats and shortenings. In assessing the harmlessness of the antioxidants, a factor of safety of at least 100 is taken. This requires that when the antioxidant is fed to experimental animals in their total diet at a level 100 times that proposed in fats for human consumption, no significant effect on growth should occur. The results in Fig. 1 are unfavorable to nordihydroguaiaretic acid and hydroquinone. Orten *et al.* (1948) noted some stunting in rats on the 1.17% level of feeding propyl gallate. No significant effect at the 1% level was noted in our rats, but retardation was significant at the 5% level. It may be noted in the figure that we have fed all the antioxidants for a lifetime with the exception of thiodipropionic acid and its esters and gum guaiac. This affords an opportunity to compare the growth effects of the various antioxidants on an equal basis. Since it required 5% of propyl gallate to affect growth in our experiments, it is believed that species difference or some other variable was responsible for the stunting of growth in the 1.17% level of the antioxidant as observed by Orten *et al.*

The dilauryl and distearyl esters of thiodipropionic acid are recommended for use in a concentration of 0.09%. These compounds represent about 0.03% of the acid and since they have been fed at a level of 3.0% without evidence of effect, the 100-times margin of safety appears to have been established.

Gum guaiac is employed in concentrations of 0.1%, and to meet the criterion about a 10% feeding level should have been carried out. Very often technical difficulties do not permit the addition of such high concentrations as would be necessary in the case of gum guaiac to establish a 100-fold margin of safety. This obstacle can be overcome in many instances by supplementary data in other species of animals. This has not been done with gum guaiac, but the long history of its use in medicine without reported injury appears to substantiate the harmlessness of the gum for antioxidant use.

2. *Other Species*

Chronic studies on propyl gallate have been conducted in guinea pigs and dogs by Orten *et al.* (1948). The guinea pigs were fed at a level of 0.011% of the antioxidant and were observed for one year. No detectable effect on the rate of growth was noted. No influence on reproduction was noted.

A group of 7 dogs was fed propyl gallate for 14 months in amounts

similar to that fed the guinea pigs. The animals on the propyl gallate regimen showed no demonstrable effects and were similar in every respect to the 5 animals which served as controls.

Thiodipropionic acid was fed to guinea pigs by Hazleton (1949) at a level of 0.5% in the drinking water and for a period of 120 days. No significant effect was noted on weight or mortality. Dogs were fed a mixture of 10 parts by weight of dilauryl thiodipropionate and one part of thiodipropionic acid and in a concentration of 0.1% and 3.0% of the acid-ester mixture in the diet. The observation period was 100 days. No untoward effects were noted.

Nordihydroguaiaretic acid has not been subjected to additional studies in dogs which is a serious omission in light of the results in rats. Bieter (1949) employed mice in one series of feeding experiments but the results were inconclusive.

Some observations on the blood constitutents were made by Orten *et al.* (1948) on rats receiving propyl gallate in the diet. The lower levels of antioxidant feeding produced no changes, but concentrations above 1.1% produced significantly lower hemoglobin values. Erythrocite and leucocyte counts were not significantly affected. Hazleton (1949) made similar blood studies on the dogs fed the acid-ester mixture of thiodipropionic acid without finding anything of significance.

In the pharmacological appraisal of an antioxidant it is important to determine the effect of heat on the stability of the substance. Reaction products or a modification of the chemical may occur which may have pharmacological effects. Orten *et al.* (1948) included such experiments in their observations. Rats were fed a heated antioxidant mixture (propyl gallate, lecithin, and corn oil) in an amount at least 100 times that which would be consumed by humans if all the dietary fat were treated with the recommended amount of antioxidant mixture effective in preserving edible fat. No detectable toxic effects were noted. Hazleton (1949) also fed thiodipropionic acid in combination with dilauryl thiodipropionate heated with lard in concentrations at least 10 times that recommended for use as an antioxidant. No injury was noted. We have conducted similar experiments with the ascorbyl palmitates without evidence of injury.

3. Mortality

The mortality rate of rats fed the several antioxidants is also an index of toxicity. A high mortality in any group of treated animals as compared with the controls indicates that the substance under test is quite toxic. If the mortality rate is about equal to that of the controls, the compound may be considered as not highly toxic. Table II summarizes

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al to that of the controls, the
y toxic. Table II summarizes

the available data on the mortality rates of the various compounds. It may be seen that none of the compounds affected the mortality rate of the rats. None of the treated groups showed significant differences from the controls in the number of animals living at any time up to the end of the experiment.

TABLE II

Mortality in Rats after 2 Years of Feeding the Several Antioxidant Diets

Antioxidant	Dosage per cent	Number of Animals Per group	Living at 2 years	Source
Nordihydroguaiaretic acid	0	18	13	Bieter (1949)
	0.1	18	12	
	0.5	18	17	
	1.0	18	12	
Nordihydroguniaretic acid	0	10	3	F & D A* (1950)
	0.1	10	1	
	0.25	10	3	
	0.5	10	3	
	1.0	10	2	
Propyl gallate	0	16	8	F & D A* (1950)
	0.1	16	4	
	0.25	16	6	
	0.5	16	6	
	1.0	16	4	
	5.0	16	3	
Thiodipropionic acid	0	20	17	Hazleton (1949)
	0.5	20	16	
	1.0	20	13	
	3.0	20	15	
Dilauryl thiodipropionate	0.5	20	10	Hazleton (1949)
	1.0	20	13	
	3.0	20	4	
Distearyl thiodipropionate	0.5	20	18	Hazleton (1949)
	1.0	20	14	
	3.0	20	18	
l-ascorbyl palmitate	0	10	4	F & D A* (1950)
	0.05	10	6	
	0.25	10	4	
d-isoascorbyl palmitate	0.05	10	4	F & D A* (1950)
	0.25	10	5	
d-isoascorbic acid	1.0	10	4	F & D A* (1950)
Gum guaiac	0	10	7	Bieter (1949)
	0.5	10	9	

TABLE II (*Continued*)

Antioxidant	Dosage per cent	Number of Animals Per group	Number of Animals Living at 2 years	Source
Catechol	0	12	2	F & D A* (1950)
	0.0625	12	4	
	0.125	12	4	
	0.25	12	1	
Catechol	0	18	5	F & D A* (1950)
	0.5	18	4	
	1.0	18	4	
Hydroquinone	0	12	2	F & D A* (1950)
	0.125	12	5	
	0.25	12	8	
	0.5	12	8	
Hydroquinone	0	18	5	F & D A* (1950)
	1.0	18	7	
	2.0	18	5	
Phenol	0	12	2	F & D A* (1950)
	0.25	12	5	
	0.5	12	6	
	1.0	12	6	

* Food and Drug Administration, unpublished data.

IV. PATHOLOGY

The predominant pathologic lesions produced by the various antioxidants after lifetime feeding to rats are summarized in Table III. If the criterion of a 100-fold margin of safety on a chronic basis is adhered to, then nordihydroguaiaretic acid and hydroquinone would be considered as deleterious substances for addition to food.

V. SUMMARY

Of the antioxidants listed in the discussion above, evidence of safety for use appears to have been established for propyl gallate, thiodipropionic acid and its dilauryl and distearyl esters, and gum guaiac. Nordihydroguaiaretic acid is not included because no experiments have been conducted in nonrodent species. Hydroquinone must be classed as a harmful and deleterious substance, and as such, has no place as an antioxidant in foods. Catechol has been proposed for antioxidant use (Olcott, 1934), but pharmacological evidence clearly classifies this chemical as a poison.

TABLE III

Pathology in Chronically Poisoned Rats (2-Year Feeding)

Substance	Diet concentration Producing injury	Predominant injury	Source
Nordihydroguaiaretic acid	0.5%	Massive cecal homorrhages with single and multiple cysts in the mesentery in the angle of junction between small and large intestine	Bieter (1949)
Nordihydroguaiaretic acid	0.5%	Inflammatory cecal lesions and slight cystic enlargement of lymph nodes near the cecum	F & D A† (1950)
Propyl gallate	1.17%	Tubular damage and presence of albuminous casts probably as the result of inanition	Orten *et al.* (1948)
Propyl gallate	5.0%	Patchy hyperplasia in the proventriculus	F & D A† (1950)
Hydroquinone	2.0%	Suggestion of increase in incidence of chronic gastrointestinal ulceration and renal tumors over spontaneous incidence	F & D A† (1950)
* *l*-ascorbyl palmitate * *d*-isoascorbyl palmitate * *d*-isoascorbic acid	0.25% 0.25% 1.0%	No lesions present attributable to the compounds fed	F & D A† (1950)
* Thiodipropionic * Dilauryl thiodipropionate * Distearyl thiodipropionate	3.0% 3.0% 3.0%	No pathological changes	Hazleton (1949)
Catechol	0.25%	Beginning hepatic cell hyperplasia	F & D A† (1950)
* Phenol	1.0%	No pathological changes	F & D A† (1950)
* Gum guaiac	0.5%	No damage attributable to the compound	Bieter (1949)

* Highest concentration fed.
† Food and Drug Administration, unpublished data.

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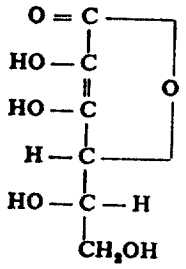
ASCORBIC ACID *

Chemical names Ascorbic acid; L-ascorbic acid; 3-keto-L-gulofurano-lactone

Synonym Vitamin C

Empirical formula $C_6H_8O_6$

Structural formula



Molecular weight 176.13

Definition Ascorbic acid, after drying over sulfuric acid for 24 hours, contains not less than 99% of $C_6H_8O_6$.

Description A white or almost white, odourless, crystalline solid. 1 g is soluble in 3.5 ml of water or 30 ml of ethanol. Insoluble in oils and fats.

Natural occurrence Ascorbic acid is present in significant amounts in a number of fruits and vegetables. It also occurs in milk and in small quantities in certain other foods.

Uses As an antioxidant in emulsions of fats and oils and as a browning inhibitor in unprocessed cut fruits, fruit pulps and juices.

SODIUM ASCORBATE

Chemical names Sodium ascorbate; sodium L-ascorbate; 3-keto-L-gulofuranolactone sodium enolate

Empirical formula $C_6H_7O_6Na$

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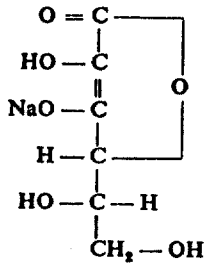
* For biological data and toxicological evaluation see pp. 20-23.

Structural formula



Molecular weight 198.11

Definition Sodium ascorbate, after drying over sulfuric acid for 24 hours, contains not less than 99% of $C_6H_7O_6Na$

Description Sodium ascorbate is a white or almost white, odourless, crystalline solid. 1 g is soluble in 2 ml of water.

Uses As an antioxidant in meat packing, the bottling industry, frozen fruit, and spice manufacturing.

Biological Data

Acute toxicity

Animal	Route [a]	LD_{50} (mg/kg body weight)	Reference
Mouse	oral	> 5000	1
"	i.v.	> 1000	1
Rat	oral	> 5000	1
"	i.v.	> 1000	1
Guinea-pig	oral	> 5000	1
"	i.v.	> 500	1

Short-term studies

Mouse

Mice given ascorbic acid orally, subcutaneously and intravenously in daily doses of 500-1000 mg/kg body weight for 7 days, showed no difference in appetite, weight gain and general behaviour from controls receiving the same amount of the biologically inactive galacturonic acid. Histological examination of various organs showed no definite changes.[1]

Guinea-pig

Guinea-pigs given ascorbic acid orally, subcutaneously and intravenously in daily doses of 400-2500 mg/kg body weight for 6 days, showed

[a] The substance was administered using an aqueous solution which was neutralized with sodium hydroxide shortly before use.

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Histological examinatio

Man

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flushing of the face, he
toxic reactions in the in

Long-term studies

No information avail

Biochemical aspects

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amounts are excreted in t
of ascorbic acid in the p
sufficient period, the pla
which a rapid urinary ex
acid occurs.[6]

Using ^{14}C-labelled asc
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Comments on experimental

From the animal studie
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However, no long-term st
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no difference in appetite, weight gain and general behaviour from controls receiving the same amounts of the biologically inactive galacturonic acid. Histological examination of various organs showed no definite changes.[1]

Man

One woman and 3 men were each given 1000 mg of ascorbic acid a day for 3 months. The ascorbic acid levels in the serum and in the white blood cells and the urinary excretion of this acid did not show any progressive changes. Further, no harmful effects were observed in these four subjects during the three months.[2]

On the other hand, after a daily dose of 5 mg/kg for 3 days a significant increase in urinary volume was observed in 30 children (10 active rheumatic, 10 convalescent rheumatic patients and 10 controls).[3] This diuretic effect was confirmed in another study on 9 patients with heart failure, given 300 mg of ascorbic acid daily.[4]

Doses up to 6000 mg of ascorbic acid were given to 29 infants, 93 children of pre-school and school age, and 20 adults for more than 1400 days. With the higher doses, toxic manifestations were observed in 5 adults and 4 infants. The signs and symptoms in adults were nausea, vomiting, diarrhoea, flushing of the face, headache, fatigue and disturbed sleep. The main toxic reactions in the infants were skin rashes.[5]

Long-term studies

No information available.

Biochemical aspects

It is well established that ascorbic acid is readily absorbed and metabolized. However, after oral administration of large quantities only small amounts are excreted in the urine, while there is a steady rise in the level of ascorbic acid in the plasma. If the oral ingestion is continued for a sufficient period, the plasma concentration rises to a maximum, after which a rapid urinary excretion of a large part of the ingested ascorbic acid occurs.[6]

Using ^{14}C-labelled ascorbic acid, it was found that in the rat after intraperitoneal injection of 1.5-5.9 mg approximately 19-29% was converted to CO_2 and only 0.4% was excreted as oxalate within 24 hours.[7]

Comments on experimental studies reported

From the animal studies it is evident that ascorbic acid is not apparently toxic after a single or a few repeated administrations of relatively large doses. However, no long-term studies have been carried out in animals to substantiate its acceptability at high dose levels.

Studies in man indicate that ascorbic acid has a diuretic effect at 5 mg/kg body weight in children and adults.[2] Certain toxic manifestations were observed in infants and adults after doses of up to 6000 mg.[4] The significance of these findings is doubtful.

Evaluation

Level causing no significant toxicological effect

A daily dose of 1000 mg taken by 4 human subjects over a period of 3 months was found to have no deleterious effect.[5] A dietary allowance of 30 mg of ascorbic acid was recommended by the League of Nations in 1938.[8] The same recommendation has been made by the Medical Research Council of Great Britain.[9]

The United States Food and Nutrition Board recommended 30 mg for infants, 75 mg for men, 70 mg for women and higher values during pregnancy and lactation. It is stated that these recommended allowances are so ample that they cover all needs of healthy individuals, even in cases where these are enhanced.[10] The minimum requirement of ascorbic acid in adults was estimated to be between 5 and 10 mg per day.[11] It is estimated that the daily intake of ascorbic acid is between 30-100 mg from natural sources. The usual therapeutic dose is 1000 mg. However, the safety of large doses of ascorbic acid over long periods of time has not been unequivocally demonstrated.

Estimate of acceptable daily intakes for man *

	mg/kg body weight
Unconditional acceptance	0-2.5
Conditional acceptance	2.5-7.5

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* The estimated daily intakes of ascorbic acid listed above do not include the ascorbic acid that is present naturally in foods.

Chemical names

Synonym

Empirical formula

Structural formula

Molecular weight

Definition

Description

Uses

Chemical names

Synonym

Empirical formula

Structural formula

* For biological data and

a diuretic effect at 5 mg/kg
toxic manifestations were
to 6000 mg.[4] The signi-

subjects over a period of
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Proc. Soc. exp. Biol., 80, 361

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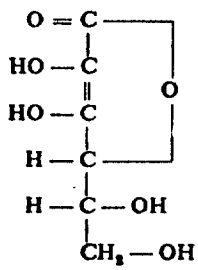
ISOASCORBIC ACID *

Chemical names Isoascorbic acid; D-isoascorbic acid; 3-keto-D-glucofuranolactone

Synonym Erythorbic acid

Empirical formula $C_6H_8O_6$

Structural formula



Molecular weight 176.13

Definition Isoascorbic acid, after drying for 24 hours over sulfuric acid, should contain not less than 98% of $C_6H_8O_6$.

Description A white or almost white, crystalline solid. 1 g is soluble in 2.5 ml of water or 20 ml of ethanol.

Uses As an antioxidant in emulsions of fats and oils and in meat and meat products, and as an inhibitor of enzymatic browning. It has no vitamin C potency.

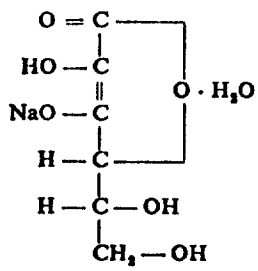
SODIUM ISOASCORBATE

Chemical names Sodium isoascorbate; sodium D-isoascorbate; 3-keto-D-glucofuranolactone sodium enolate monohydrate

Synonym Sodium erythorbate

Empirical formula $C_6H_7O_6Na.H_2O$

Structural formula



* For biological data and toxicological evaluation see pp. 24-25.

Molecular weight	216.13
Definition	Sodium isoascorbate contains not less than 98% of $C_6H_7O_6Na.H_2O$
Description	Sodium isoascorbate is a white, almost odourless crystalline solid. 1 g is soluble in 2 ml of water.

Biological Data

Acute toxicity

No information available.

Short-term studies

Rat

Groups of 10 male rats were fed for 36 weeks on diets containing 1% of isoascorbic acid, and on diets without isoascorbic acid. There was no difference between the treated rats and the controls with respect to rate of growth and mortality. Gross post-mortem examination and microscopic studies of various organs revealed no lesion attributable to isoascorbic acid.[1]

Long-term studies

Rat

Groups of rats were fed on diets containing 1% of isoascorbic acid and diets without isoascorbic acid for 2 years. The growth rate, mortality, and histopathology were not affected by the treatment.[2]

Biochemical aspects

Isoascorbic acid is readily absorbed and metabolized. Following an oral dose of 500 mg of isoascorbic acid to human subjects the blood level curves for ascorbic acid and isoascorbic acid showed a similar rise. In 5 human subjects, an oral dose of 300 mg was shown to have no effect on the urinary excretion of ascorbic acid.[3] Isoascorbic acid was found to have no antagonistic effect on the action of ascorbic acid.[4]

Comments on experimental studies reported

The short-term and long-term studies in rats were thorough. However, no long-term studies have been carried out in other species. The biochemical studies in man indicate that isoascorbic acid is readily metabolized and does not affect the urinary excretion of ascorbic acid.

Level causing no signif

1% (= 10 000 p.p.n
per day.

Estimate of acceptable

Unconditional
Conditional a

1. Fitshugh, O. G. & Nels
2. Lehman, A. J., Fitzhug
Food Res., 3, 197
3. Kadin, H. & Osadca, N
4. Gould, D. S. (1948) *Ar*

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Chemical names	As to
Synonym	Vi
Empirical formula	C_{2}
Structural formula	O HO HO H HO
Molecular weight	41
Definition	Asc C_{2}
Description	A odc
Use	As

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of ascorbic acid.

Evaluation

Level causing no significant toxicological effect in the rat

1% (= 10 000 p.p.m.) in the diet, equivalent to 500 mg/kg body weight per day.

Estimate of acceptable daily intakes for man

	mg/kg body weight
Unconditional acceptance	0-2.5
Conditional acceptance	2.5-7.5

References

1. Fitshugh, O. G. & Nelson, A. A. (1946) *Proc. Soc. exp. Biol.*, 61, 195
2. Lehman, A. J., Fitzhugh, O. G., Nelson, A. A. & Woodward, G. (1951) *Advanc. Food Res.*, 3, 197
3. Kadin, H. & Osadca, M. (1959) *J. Agric. Food Chem.*, 7, 358
4. Gould, D. S. (1948) *Arch. Biochem.*, 19, 1

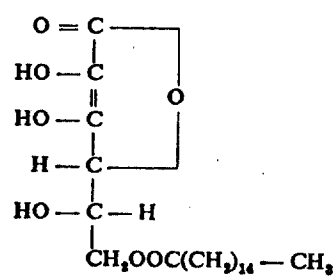
ASCORBYL PALMITATE

Chemical names Ascorbyl palmitate; L-ascorbyl palmitate; 6-palmitoyl-3-keto-L-gulofuranolactone

Synonym Vitamin C palmitate

Empirical formula $C_{22}H_{38}O_7$

Structural formula



Molecular weight 414.55

Definition Ascorbyl palmitate contains not less than 98% of $C_{22}H_{38}O_7$

Description A white or yellowish-white solid, with a citrus-like odour. 1 g is soluble in 4.5 ml of ethanol.

Use As an antioxidant.

022

Diuretic action of sodium ascorbate; observations on animals and on man

V. Cagli - G. P. Cantore - J. Pecori Giraldi - M. Virno

In 1954, reporting the results obtained in the treatment of acute infectious hepatitis by intravenous administration of 10 g of ascorbic acid, Baur and Staub (1) mentioned, among other things, an "increase of diuresis." In 1957, Kirchmair and Kirsch (4), giving the same dose to children over 5 (and half that dose to children of younger age) suffering from acute viral hepatitis, also mentioned "abundant diuresis."

It should be noted that the above papers by the German authors represent the first mention of the use of ascorbic acid (sodium salt) at such high doses. Consider that the average requirement of ascorbic acid as vitamin is stated to be for the normal adult 70-75 mg and that for use in states of vitamin deficiency doses of 120 mg and up to a maximum of 1 g per day are recommended (3).

Further we should recall that quite recently Del Piano and Lisi (2) gave doses of 1 g per 5 kg of body weight in order to study the response of bilirubinemia in various forms of hepatic lesions.

It must be stressed that all authors find perfect tolerance of the drug at these doses and absence of any inconvenience.

On the basis of these observations, we proposed to find out if the doses of sodium ascorbate could be further increased in order to obtain a diuretic effect os such proportions that it could be useful in therapy.

Materials and methods

In all our experiments, on animals and on man, weused ascorbic acid in 20% solution brought to pH 7.4 with sodium bicarbonate.

Observations on the animal

Acute toxicity: Acute toxicity was studied in the rabbit, by injection into the marginal vein of the ear.

Chronic toxicity: Chronic toxicity was studied in the dog with rapid daily

injection of sodium ascorbate - 1 g/kg i.v. - for 20 consecutive days. The animal was then sacrificed with a lethal dose of curare (gallamine triiodide) and the kidney and liver were taken out for histological examination.

Recording of the arterial pressure: Continuous recording of the arterial pressure was carried out on the rabbit by applying a cannula in the femoral artery under local anesthesia (1% scurocaine). The animal was immobilized with curare (gallamine triiodide: 4 mg/kg i.v. and 40 mg/kg i.m.) to avoid having to use a general anesthetic which might have interfered with the interpretation of the results and subjected to controlled respiration, after having practiced tracheotomy.

Diuresis: Simultaneously with the recording of the arterial pressure, the diuresis was measured in the rabbit. The urine was collected by insertion of a catheter.

The rate of infusion of the sodium ascorbate solutions was 5 ml/minute in the marginal vein of the rabbit's ear.

Observations on man

The 20% sodium ascorbate solutions at pH 7.4 for the clinical tests were sterilized by a GD 15 Membrane Filter (Membranfilter - Göttingen). The solutions were administered to the patients at a rate of 60 drops per minute. In all cases we determined the hematocrit, the natriemia and the potassiemia (the latter two by flame spectrophotometer) before the treatment, 1 hour after end of infusion, and 24 hours after end of experiment. There were determined also the glycemia and the serum urea (enzymatic method with urease) before treatment and 1 and 24 hours after treatment. In addition, the body weight, the arterial pressure, the volume of urine excreted in the 24 hours before the infusion and at certain intervals during the 24 hours following the treatment were determined. The urine was examined also for its sodium and potassium content and for the pH variations. The fluid intake/output and the complete water-salt metabolism of each patient was checked during the 24 hours before the infusion and during the 24 hours following the treatment.

In three patients there was determined also the ascorbinemia before and 1

and 24 hours after the treatment, as well as the quantity of ascorbic acid excreted with the urine at the same intervals of time (King's method with dichlorophenolindophenol).

Results

In the animals

The acute toxicity tests made in the rabbit intravenously at an infusion rate of 5 ml/minute showed that up to a dose of 5.3 g/kg i.v. no toxic effect occurred. At doses of 6.4 g/kg i.v. tremors in the limbs were observed about one hour after the injection. At doses of 7.3 g/kg i.v., the animal died about 5 hours after administration, with convulsive attacks; 8.5 g/kg i.v. were lethal for the rabbit after 1 h; 10.4 g/kg i.v., 40 minutes after the injection.

In five rabbits 30 minutes before administration of 8.5 g/kg i.v. of sodium axcorbate, 50 ml/kg of physiological solution were applied with a probe introduced into the stomach; instead of dying in about 1 hour, the animals then survived for 2 hours.

Doses of 1 g/kg i.v. of sodium ascorbate injected in the dog for 20 consecutive days caused no symptom in the animal. Only a change in body weight was found, which increased in 20 days from 6.200 kg to 8.400 kg. When the animal was sacrificed on the twentieth day, the liver as well as the kidney were found to be morphologically normal in the histological examination.

From the tests made in the rabbit on the arterial pressure at doses of 0.5-1 g/kg i.v. of the 20% sodium ascorbate solution it resulted that with a solution at pH 7.4 the arterial pressure of the animal underwent no significant variations, while moving away from these pH values, substantial modifications of arterial pressure appeared in the animal.

The diuretic effect of sodium ascorbate given intravenously at the dose of 1.5 g/kg was studied in a series of 6 rabbits together with continuous recording of the arterial pressure. In all animals an impressive diuresis was found, which reached average values of 73.08 ml of urine within 1 hour from the administration. Under the same test conditions, a group of 6 rabbits received a dose of 1.5 g/kg of 20% mannitol intravenously. We chose mannitol for this

comparison because its intense activity as osmotic diuretic is known. The average quantity of urine excreted by the rabbits within 1 hour from the administration of mannitol was 36.33 ml (fig. 1).

The application of larger or smaller doses than 1.5 g/kg i.v. of sodium ascorbate caused in the rabbits a diuretic effect proportional to the dosage given.

Fig. 1. Comparison between the diuretic action of sodium ascorbate and of mannitol (mean of 6 animals for each substance). Time after the administration (minutes).

Fig. 2. Diuretic action of 1 g/kg sodium ascorbate i.v. in man (mean of 6 patients). Time after the administration (hours).

In man

In view of the perfect tolerance of sodium ascorbate given intravenously in the animal up to 5 g/kg, we felt that clinical experimentation would be permissible.

The subjects treated were patients hospitalized for brain tumors in the Clinic for Nervous and Mental Diseases of the University, none of them presented edemata or any major alteration of the electrolytic fluid equilibrium. After initiating the tests at doses of 200 mg/kg, we progressively increased the quantity of the drug to 1 g/kg i.v. Also at these doses the drug was tolerated perfectly locally as well as generally, as long as the pH of the solution stayed at 7.4 and the rate of application did not exceed 60 drops per minute.

The diuresis underwent a clear and constant increase, equal to about 100% calculated as an average. The greatest increase occurred within the first 6 hours after the end of the infusion (Table 1; fig. 2).

In the serum, a clear and constant reduction of the potassium was observed. This phenomenon did not match up exactly in the individual case with a parallel increase of the potassic diuresis (Table 1).

The sodium of the serum remained practically unchanged, while the sodic diur-

esis increased obviously in proportion with the sodium present in the infustion liquid (Table 1).

The sodiuria after infusion showed values perfectly in line with the quantities of sodium applied (Table 2).

The hematocrit presented a slight tendency to decrease which, while not significant, assures the absence of a state of extracellular dehydration (Table 1).

Table 1. Effects of intravenous administration of 1 g/kg of sodium ascorbate.

Patient	Quantity		Specific gr.		pH		Na m.eq o/oo		K m.eq o/oo	
	Before	After	Before	After	Before	After	Before	After	Before	After
A) on human urine										
M.A.	1,350	2,250	1,020	1,020	7.8	8.1	215	281	623	643
A.Z. (*)	3,300	5,170	1,030	1,032	6.6	6.6	110	670	473	1,787
F.A.	1,250	2,450	1,022	1,032	7.2	6.6	197	392	54	63
R.I.	1,100	2,550	1,032	1,020	6.9	6.6	130	523	69	68
C.H.	1,100	1,830	1,028	1,012	6.4	7.2	88	146	23	43
I.E.	950	3,000	1,008	1,038	7	7	115	1,267	136	910
Mean	1,508	2,875					142	546	229	585
Mean (**)		1,150	2,416				149	521	181	343

Patient	Na (serum) m.eq o/oo			K (serum) m.eq o/oo			Hematocrit		
	Before	After 1 h	After 24 h	Bef.	After 1 h	After 24 h	Bef.	After 1 h	After 24 h
B) on human blood									
M.A.	141.6	141.6	136.9	5.1	5.1	3.8	19	21	19
Z.A. (*)	139.1	160.4	147.8	5.6	5.6	2.5	47	50	45
F.A.	141.6	149.1	143.4	--	--	5.3	42	40	41
R.I.	121.5	121.5	126	4.8	4.8	4.3	30	26	28
C.H.	96.9	105	93.4	6.1	6.4	3	37	39	38

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Table continued.

I.E.	124.5	128.2	124.5	--	--	4.6	47	45	42
Mean	127.5	140.9	128.6	5.4	5.4	3.9	39	37	39
Mean (**)	125.2	129	124.8	5.3	5.4	3.5	37	34	33

(*) The patient was treated simultaneously with glycerol (Luxoral).

(**) Mean of the values excluding the patient marked (*).

The pH of the urine showed slight and inconsistent modifications; but it is important to point out that on the whole it remained at values of weak acidity (Table 1).

The ascorbinemia increased rapidly and returned to the starting values after 24 hours; the elimination with the urine within the first 24 hours is practically total for doses of 0.5 g/kg, while it is slightly more than half at doses of 1 g/kg.

Table II. Urinary elimination of sodium after administration of sodium ascorbate.

Patient	Basal sodiuria (g)	Sodiuria after infusion (g)	Sodium given (g)	Increase of sodiuria (g)
M.A.	4.96	6.48	6.35	1.52
Z.A.	2.54	15.43	15.86	12.89
F.A.	4.55	9.02	8.33	4.47
C.H.	2.04	3.37	4.76	1.33
I.E.	2.65	29.16	15.86	26.51
Mean	3.29	12.74	10.64	9.46

We must lastly report on the response of the ureic nitrogen of the blood and on the glycemia. As to the ureic nitrogen, we do not list the data because they always remained unchanged from the normal values. But as far as the glycemia is concerned, we must state that, while the values 24 hours after the infusion were consistently coincident with the starting values, in the

determinations made two hours after the end of the infusion great modifications were observed. These modifications appeared in one or the other sense depending on the method employed in the analysis. With the enzymatic method the values were abnormally low, coming very close to zero; with the Nelson-Somogyi method the values were between 1.60 and 3.00 o/oo. The result obtained with the Nelson-Somogyi method can easily be explained: Because of its strongly reducing properties, the ascorbic acid leads to a reduction of the cupric to cuprous sulfate, hence the false increase of the glycemic values. As to the results obtained with the enzymatic method, on the other hand, all we can say is that this is simply a phenomenon of interference (in substance, falsely reduced values), so much so that the addition in vitro of ascorbic acid to a standard solution of glucose makes it undeterminable. This probably occurs by interference, due to the reducing power of the ascorbic acid, on the glucose-oxidase enzyme which brings about the reaction: Glucose + O_2 --: Gluconic acid + H_2O_2, and/or on the peroxidase capable of transforming the H_2O_2, in the presence of an oxygen acceptor, in the colored compound which is being analyzed.

Conclusions

The results obtained by us in animals and in man clearly show that sodium ascorbate at the doses and in the form of application employed is an efficient diuretic. As far as we known, nobody before us has used such high doses of ascorbic acid (1 g/kg) in clinical medicine, but we have found that such doses can be used in man as they are quite far from those letahl to the test animal (7 g/kg i.v. in the rabbit); also the chronic toxicity in the dog was found to be zero at these doses (1 g/kg i.v.). The clinical use has completely confirmed the observations on the animals as to the perfect local and general tolerance of the drug.

The degree of diuretic action is considerable if we consider that the diuresis is on the average doubled and that the diuretic effect in the animal is doubled in relation to that obtainable with equal doses of mannitol, which is one of the most widely used osmotic diuretics.

As to the mechanism of action, we must examine separately the action of the

ascorbic acid and that of the sodium bicarbonate. As far as the ascorbic acid is concerned, the first mechanism envisioned is that of an osmotic action. It is suggested by two considerations: (a) The extremely high doses employed; (b) the delay in the death of the animal which has been given a lethal dose if a fluid charge has been given before. It might be objected that the observations of the German authors (1, 4), according to whom a diuretic effect is observed (if of unspecified degree) in subjects treated only with 10 g of ascorbic acid (sodium salt), make an osmotic mechanism not very likely. It must be considered however, that in those studies patients with acute hepatitis were concerned and that the increase of the diuresis was accompanied by improvement of the disease (reduction of the bilirubinemia, subjective improvement, etc.) so that the evaluation must take into account that also in the spontaneous development, when a patient suffering from infectious hepatitis begins to get better, this is marked by the release of the diuresis. In confirmation of a mechanism of an osmotic type we can state that at doses smaller than 1 g/kg to 0.5 g/kg we have observed no significant increase of the diuresis either in animals or in man.

Regarding the action of sodium bicarbonate, however, it must be recalled that this salt is found to have diuretic properties attributable to the carbon ion (HCO_3^-), which is normally resorbed by the proximal tubule following transformation into H_2CO_3; if the H^+ ions necessary for that transformation are insufficient in quantity with respect to the excess of HCO_3^-, the latter is excreted as $NaHCO_3$ and entails an iso-osmotic quantity of water, hence the increase of the diuresis (3). But in our case it is considered that the addition of sodium bicarbonate to the ascorbic acid leads to a lively effervescence and thus to the elimination of CO_2, so that it seems to us one cannot postulate the intervention of HCO_3^- in the diuretic mechanism.

The only important metabolic effect found was the reduction of the potassemia which, in the individual cases, did not always correspond to an increase of the potassiuria. However, we are dealing with an experimentation carried out on patients with an already increased basal potassiuria, and anyway it is still too limited to permit final conclusions. We believe, therefore, only hypoth-

eses can be advanced. Reduction of the potassiemia can occur, apart from the cases of insufficient intake or gastro-enteric loss which arenot of interest here, due to:

a) loss with the urine by:

1) block K^+ resorption in the proximal tubule (as with the thiazidic diuretics); in our case such a mechanism is entirely hypothetic;

2) metabolic alkalosis induced by the introduction of a salt of a weak acid and a strong base which, imposing the recovery in the tubule of the maximum quantity of H^+, leads, parallelly, to increased K^+ excretion;

b) Entrance of the potassium in the cells - to be hinted at only quite hypothetically, the more so as the alkalosis is more likely to create the conditions for a release of potassium from the cells than for its entrance (5).

In conclusion, all be it with the reserves due to the incompleteness of our observations, we believe that in the present state the most probable mechanism of the demonstrated diuretic action of sodium ascorbate must be identified with an osmotic diuresis.

Summary

(See pp. 841-2 of original publication)

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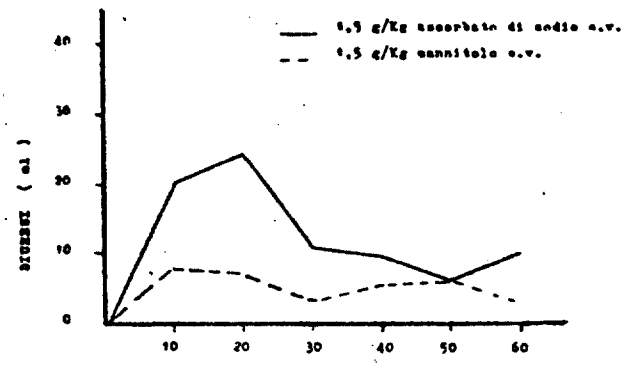

Figure 1

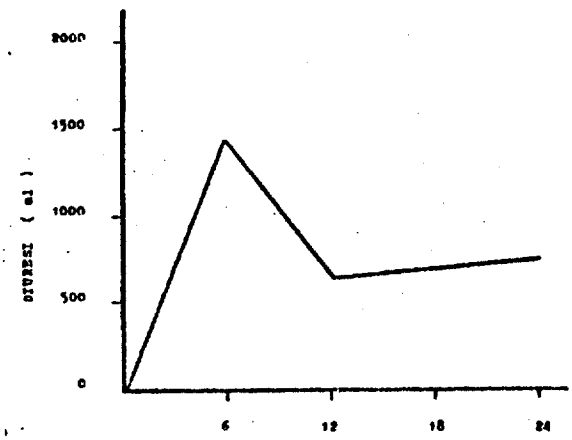

Figure 2

Summary

Summary

Gagli V., Cantore G. P., Pegori Giraldi J., Virno M., *On the diuretic action of the ascorbic acid (sodium salt).*

The intravenous administration of ascorbic acid (sodium salt) at doses of g 1/kg of body weight was shown to produce a marked diuretic effect in man and in the animal.

Though the doses employed were high, they proved to be very distant from lethal ones and, practically, were well tolerated by man and the animal.

The only important metabolic effect observed following the drug's administration was the decrease of kaliemia, which was not always accompanied by a proportional increase of kaliuria.

The AA. claim that an osmotic mechanism would probably account for the diuresis induced by this salt.

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AZIONE DIURETICA DELL'ASCORBATO DI SODIO: OSSERVAZIONI SUGLI ANIMALI E SULL'UOMO

V. CAGLI - G. P. CANTORE - J. PECORI GIRALDI - M. VIRNO

Nel 1954 Baur e Staub (1), comunicando i risultati ottenuti nel trattamento della virus-epatite acuta con la somministrazione per via endovenosa di g 10 di acido ascorbico, segnalavano, tra l'altro, un « aumento della diuresi ». Nel 1957 Kirchmair e Kirsch (4), somministrando la stessa dose a bambini oltre i 5 anni (e una dosa dimezzata in bambini di età inferiore) affetti da epatite acuta virale, segnalavano anch'essi una « diuresi abbondante ».

E' da rilevare come i ricordati lavori degli AA. tedeschi rappresentino le prime segnalazioni relative all'impiego dell'acido ascorbico (sale sodico) a dosi così elevate. Si consideri che il fabbisogno medio dell'acido ascorbico come vitamina viene indicato per l'adulto normale in mg 70-75 e che per l'impiego negli stati carenziali si indicano dosi di mg 120 e come massimo di g 1 *pro die* (3).

Dopo le segnalazioni già citate, dobbiamo ricordare che del tutto recentemente Del Piano e Lisi (2) hanno somministrato dosi di g 1/5 kg peso allo scopo di studiare l'andamento della bilirubinemia in epatopazienti di diversa natura.

E' importante sottolineare che tutti gli AA. sono concordi nel rilevare la perfetta tollerabilità del farmaco a queste dosi e l'assenza di qualsiasi inconveniente.

Partendo dalle osservazioni ricordate, ci siamo proposti di stabilire se fosse possibile aumentare ulteriormente le dosi di ascorbato di sodio ed ottenere così un effetto diuretico di proporzioni tali da poter essere utilmente sfruttato in terapia.

Materiali e metodi

In tutte le nostre esperienze, sia nell'animale che nell'uomo, abbiamo usato l'acido ascorbico in soluzione al 20% portato a pH 7.4 con bicarbonato di sodio.

Osservazioni sull'animale

Tossicità acuta: la tossicità acuta è stata studiata nel coniglio per via endovenosa, mediante iniezione nella vena marginale dell'orecchio.

Tossicità cronica: la tossicità cronica è stata studiata nel cane con iniezione rapida giornaliera di ascorbato di sodio — g 1/kg e.v. per la durata di 20 giorni consecutivi. L'animale è stato quindi sacrificato con una dose letale di curaro (trijoduro di gallamina) e sono stati prelevati il rene e il fegato per l'esame istologico.

Registrazione della pressione arteriosa: la registrazione in continuo della pressione arteriosa è stata effettuata nel coniglio mediante incannulamento dell'arteria femorale in anestesia locale (scurocaina all'1%). L'animale veniva immobilizzato con curaro (trijoduro di gallamina: mg 4/kg e.v. e mg 40/kg i.m.) in modo da non dover ricorrere ad un anestetico generale che avrebbe potuto interferire con l'interpretazione dei risultati, e sottoposto a respirazione controllata, dopo aver praticato la tracheotomia.

Diuresi: contemporaneamente alla registrazione della pressione arteriosa, veniva misurata

Il lavoro spetta in parti eguali agli AA.

034

PER LE NISTRONE
ARTERIOSCLEROSI
PROTEGGE
VASORUTINA FORTE
INAM

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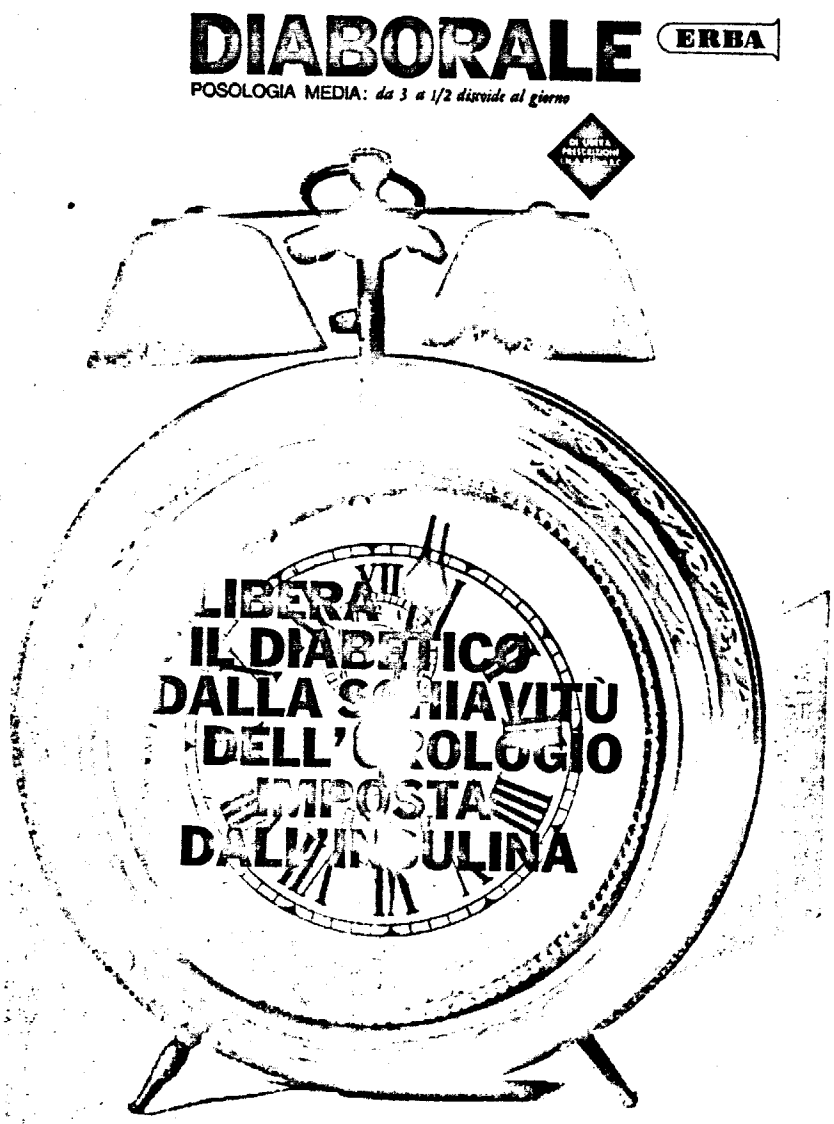

l coniglio la diuresi. Le urine venivano raccolte mediante inserimento di catetere vescicale permanenza.

La velocità di infusione delle soluzioni di corbato di sodio erano di 5 ml/minuto nella na marginale dell'orecchio di coniglio.

sservazioni sull'uomo

Le soluzioni di ascorbato di sodio al 20% pH 7,4 per le prove cliniche sono state steilizzate mediante Filtro a membrana GD 15 Membranfilter - Göttingen). Le soluzioni venino somministrate ai pazienti ad una velocità 60 goccie al minuto. In tutti i casi sono ate determinate l'ematocrito, la natriemia, la otassiemia (queste ultime mediante spettrofonetro a fiamma) prima del trattamento, dopo ora dal termine dell'infusione e dopo 24 ore alla fine dell'esperienza. Sono state inoltre determinate la glicemia e l'urea sierica (metodo enzimatico all'ureasi), prima, 1 ora e 24 ore dopo il trattamento. Oltre a queste ricerche, si proceduto alla determinazione del peso corporeo, della pressione arteriosa, del volume delle urine escrete nelle 24 ore precedenti la infusione e a determinati intervalli nell 24 ore seguenti al trattamento. Le urine venivano anche esaminate per il contenuto di sodio e potassio e per le variazioni di pH. Il bilancio idrico il ricambio idro-salino completo di ogni paziente è stato tenuto sotto controllo nelle 24 ore precedenti la infusione e nelle 24 ore seguenti l trattamento.

In tre malati, è stata inoltre determinata ascorbinemia prima e 1 ora e 24 ore dopo il attamento, come pure la quantità di acido scorbico escreta con le urine agli stessi intervalli di tempo (metodo di King al diclorofeno-indofenolo).

ISULTATI

egli animali

Le prove di tossicità acuta effettuate nel oniglio per via endovenosa ad una velocità di fusione di 5 ml/minuto, hanno messo in evidenza che fino ad una dose di 5,3 g/kg e.v. on si verificava alcun effetto tossico. Alle dosi di 6,4 g/kg e.v. si osservavano dopo circa un'ora dall'iniezione tremori agli arti. Alle dosi di 7,3 g/kg e.v., l'animale moriva dopo circa 5 ore dalla somministrazione con attacchi convulsivi; 8,5 g/kg e.v. erano letali per il coniglio dopo 1 h; 10,4 g/kg e.v. dopo 40 minuti dall'iniezione.

In cinque conigli, 30 minuti prima della somministrazione di 8,5 g/kg e.v. di ascorbato di sodio, veniva praticato un carico idrico — 50 ml/kg di soluzione fisiologica per via orale — mediante sonda introdotta nello stomaco; in queste condizioni gli animali, anziché morire in circa 1 ora, sopravvivevano per 2 ore.

Le dosi di 1 g/kg e.v. di ascorbato di sodio iniettate nel cane per 20 giorni consecutivi non provocavano nessun sintomo nell'animale. Si constatava solo una variazione del peso corporeo che in 20 giorni aumentava da kg 6,200 a kg 8,400. Sacrificato l'animale al ventesimo giorno, all'esame istologico sia il fegato che il rene risultavano morfologicamente normale.

Dalle prove effettuate nel coniglio sulla pressione arteriosa alle dosi di 0,5-1 g/kg e.v. della soluzione di ascorbato di sodio al 20%, risultava come con soluzione a pH 7,4, la pressione arteriosa dell'animale non subiva variazioni significative, mentre allontanandosi da questi valori di pH, si manifestavano nell'animale sensibili modificazioni di pressione arteriosa.

L'effetto diuretico dell'ascorbato di sodio somministrato per via endovenosa alla dose di 1,5 g/kg, è stato studiato in una serie di 6 conigli unitamente alla registrazione in continuo della pressione arteriosa. In tutti gli animali si è verificata una imponente diuresi che raggiungeva valori medi di 73,08 ml di urina nel periodo di 1 h dalla somministrazione. Un gruppo di 6 conigli nelle stesse condizioni sperimentali, ricevette, invece, una dose di 1,5 g/kg di mannitolo al 20% per via endovenosa. Abbiamo scelto il mannitolo per questo confronto, in quanto ne è nota l'intensa attività come diuretico osmotico. La media delle quantità di urine escrete dai conigli in 1 ora dalla somministrazione di mannitolo era di 36,33 ml (fig. 1).

Le somministrazioni di dosi maggiori o minori di 1,5 g/kg e.v. di ascorbato di sodio provocavano nei conigli un effetto diuretico riferibile e proporzionale al dosaggio praticato.

Nell'uomo

La perfetta tollerabilità dimostrata dall'ascorbato di sodio per somministrazione fino a g 5/kg nell'animale per via endovenosa ci ha fatto ritenere consentita la sperimentazione clinica.

I soggetti sottoposti al trattamento erano pazienti ricoverati presso la Clinica di Malattie Nervose e Mentali dell'Università perché affetti da tumori endocranici: nessuno di essi presentava edemi o comunque importanti alterazioni dell'equilibrio idrico elettrolitico. Dopo avere iniziato le prove con dosi di mg 200/kg abbiamo progressivamente aumentato la quantità del farmaco fino a raggiungere 1 g/kg endovenoso. Anche a queste dosi, il farmaco era perfettamente tollerato sia dal punto di vista locale che generale, sempre a condizione che il pH della soluzione non si discostasse da valori di 7,4 e che la velocità di somministrazione non superasse le 60 goccie al minuto.

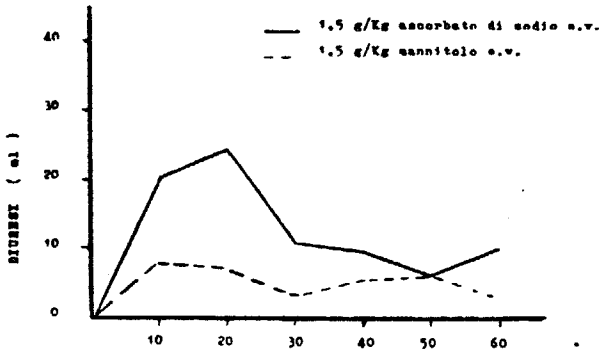

Fig. 1 - Confronto tra l'azione diuretica dell'ascorbato di sodio e del mannitolo (media di 6 animali per ciascuna sostanza) - Tempo dopo la somministrazione (minuti).

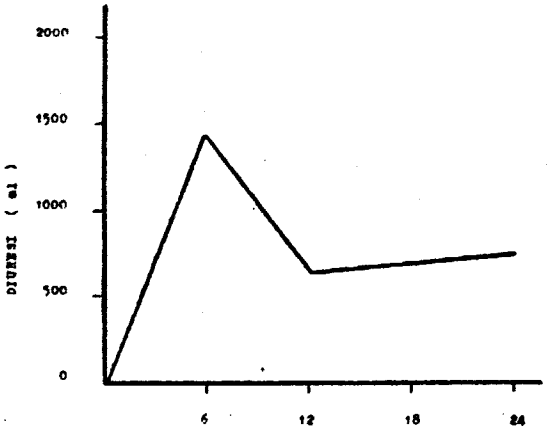

Fig. 2 - Azione diuretica dell'ascorbato di sodio 1 g/kg e.v. nell'uomo (media di 6 pazienti) - Tempo dopo la somministrazione (ore).

La diuresi ha subìto un netto e costante incremento che calcolato nella media è pari circa al 100%. Il massimo dell'incremento si ebbe entro le prime 6 ore dalla fine dell'infusione (tab. 1; fig. 2).

Nel siero si osservava netta e costante diminuzione del potassio: tale fenomeno non trova nel singolo caso esatta corrispondenza con un parallelo aumento della diuresi potassica (tab 1).

Il sodio del siero restava praticamente invariato mentre aumentava la diuresi sodica in rapporto evidentemente con il sodio presente nel liquido di infusione (tab. 1).

La sodiuria dopo l'infusione era su valori perfettamente corrispondenti alla quantità di sodio somministrata (tab. 2).

L'ematocrito presentava una lieve tendenza alla diminuizione che, anche se non significativa, garantisce però la mancanza di uno stato di disidratazione extracellulare (tab. 1).

TABELLA I - *Effetti della somministrazione endovenosa di 1 g/k di ascorbato di sodio.*

Paziente	Quantità prima	Quantità dopo	Peso specifico prima	Peso specifico dopo	pH prima	pH dopo	Na mEq ‰ prima	Na mEq ‰ dopo	K mEq ‰ prima	K mEq ‰ dopo
A) sulle urine dell'uomo										
M. A.	1.350	2.250	1.020	1.020	7,8	8,1	215	281	623	643
Z. A. (*)	3.300	5.170	1.030	1.032	6,6	6,6	110	670	473	1.787
F. A.	1.250	2.450	1.022	1.032	7,2	6,6	197	392	54	63
R. I.	1.100	2.550	1.032	1.020	6,9	6,6	130	523	69	68
C. H.	1.100	1.830	1.028	1.012	6,4	7,2	88	146	23	43
I. E.	950	3.000	1.008	10.38	7	7	115	1.267	136	910
Medie	1.508	2.875					142	546	229	585
Medie (**)	1.150	2.416					149	521	181	343

Paziente	Na (siero) mEq ‰ prima	Na (siero) mEq ‰ dopo 1 h	Na (siero) mEq ‰ dopo 24 h	K (siero) mEq ‰ prima	K (siero) mEq ‰ dopo 1 h	K (siero) mEq ‰ dopo 24 h	Ematocrito prima	Ematocrito dopo 1 h	Ematocrito dopo 24 h
B) sul sangue dell'uomo									
M. A.	141,6	141,6	136,9	5,1	5,1	3,8	19	21	19
Z. A. (*)	139,1	160,4	147,8	5,6	5,6	2,5	47	50	45
F. A.	141,6	149,1	143,4	—	—	5,3	42	40	41
R. I.	121,5	121,5	126	4,8	4,8	4,3	30	26	28
C. H.	96,9	105	93,4	6,1	6,4	3	47	39	38
I. E.	124,5	128,2	124,5	—	—	4,6	47	45	42
Medie	127,5	140,9	128,6	5,4	5,4	3,9	39	37	39
Medie (**)	125,2	129	124,8	5,3	5,4	3,5	37	34	33

(*) Il paziente era trattato contemporaneamente con glicerolo (Luxoral).
(**) Media dei valori con esclusione del paziente contrassegnato con (*).

Il pH urinario presentava lievi ed incostanti modificazioni: è però importante rilevare come esso si mantenga per lo più sui valori di debole acidità (tab. 1).

L'ascorbinemia si elevava rapidamente e tornava ai valori di partenza dopo 24 ore; l'eliminazione con le urine entro le prime 24 ore è praticamente totale con dosi di g 0,5/kg, mentre è di poco superiore alla metà con dosi di g 1/kg.

TABELLA II - *Eliminazione urinaria del sodio dopo somministrazione di ascorbato di sodio.*

Paziente	Sodiuria di base (g)	Sodiuria dopo infusione (g)	Sodio somministrato (g)	Incremento della sodiuria (g)
M. A. . .	4,96	6,48	6,35	1,52
Z. A. . .	2,54	15,43	15,86	12,89
F. A. . .	4,55	9,02	8,33	4,47
R. I. . .	3,00	12,03	12,70	9,03
C. H. . .	2,04	3,37	4,76	1,33
I. E. . .	2,65	29,16	15,86	26,51
Media . .	3,29	12,74	10,64	9,46

Dobbiamo infine riferire sul comportamento dell'azoto ureico del sangue e della glicemia. Per ciò che concerne l'azoto ureico, abbiamo omesso di tabulare i dati perché essi sono sempre rimasti immodificati sui valori normali. Per quanto si riferisce, invece, alla glicemia, dobbiamo dire che, mentre i valori a 24 ore di distanza dall'infusione erano costantemente sovrapponibili a quelli di partenza, nelle determinazioni eseguite a due ore di distanza dal termine dell'infusione stessa si osservavano invece importanti modificazioni. Tali modificazioni si presentavano in un senso o nell'altro a seconda della metodica impiegata nel dosaggio. Con il metodo enzimatico i valori erano abnormemente bassi, fino a valori molto vicini allo zero; con il metodo Nelson-Somogyi i valori erano compresi tra g 1,60 e 3,00‰. E' facilmente spiegabile il risultato ottenuto con il metodo di Nelson-Somogyi: infatti l'acido ascorbico per le sue energiche proprietà riducenti porta ad una riduzione del solfato rameico a rameoso, onde il falso aumento dei valori glicemici. Per quanto si riferisce invece ai risultati ottenuti con il metodo enzimatico possiamo soltanto dire che si tratta di un semplice fenomeno di interferenza (in sostanza di valori *falsamente* diminuiti) tanto è vero che l'aggiunta *in vitro* di acido ascorbico ad una soluzione standard di glucosio lo rende indisabile. Ciò accade verosimilmente per interferenza, dovuta al potere riducente dell'acido ascorbico, sull'enzima glucosio-ossidasi che provoca la reazione: glucosio + O_2 → ac. gluconico + H_2O_2, e/o sulla perossidasi capace di trasformare l'H_2O_2, in presenza di un accettore di ossigeno, nel composto colorato che viene dosato.

CONCLUSIONI

I risultati da noi ottenuti negli animali e nell'uomo dimostrano con evidenza come l'ascorbato di sodio alle dosi e per la via di somministrazione impiegata sia un efficace diuretico. A quanto ci consta nessuno ha impiegato prima di noi in clinica dosaggi altrettanto elevati di acido ascorbico (g 1/kg), ma tali dosaggi ci sono sembrati utilizzabili nell'uomo perché assai distanti da quelli letali per l'animale da esperimento (g 7/kg e.v. nel coniglio); anche la tossicità cronica nel cane si è dimostrata nulla a queste dosi (g 1/kg e.v.): l'impiego clinico ha completamente confermato le osservazioni sugli animali, quanto alla perfetta tollerabilità locale e generale del farmaco.

L'entità dell'azione diuretica è notevole; si consideri che la diuresi viene in media raddoppiata e che l'effetto diuretico nell'animale è doppio rispetto a quello ottenibile con pari dosi di mannitolo che rappresenta uno dei diuretici osmotici più impiegati.

Quanto al meccanismo di azione è necessario prendere in esame separatamente l'intervento dell'acido ascorbico e quello del bicarbonato di sodio. Per quanto si riferisce all'acido ascorbico, il primo meccanismo che si prospetta è quello di un'azione osmotica. Tale meccanismo è suggerito da 2 considerazioni: *a*) le fortissime dosi impiegate; *b*) il ritardo nella morte dell'animale cui si somministri una dose letale se è stato fatto precedentemente un carico idrico. Si potrebbe obiettare che le osservazioni degli AA. tedeschi (1, 4) secondo le quali si osserva un effetto diuretico (peraltro di entità imprecisata) in soggetti trattati con soli g 10 di acido ascorbico (sale sodico) rendon poco verosimile un meccanismo osmotico. Bisogna però considerare che si trattava, in quelle ricerche, di pazienti affetti da epatite acuta e che l'aumento della diuresi si accompagnava a miglioramento della malattia (diminuzione della bilirubinemia, miglioramento soggettivo, ecc.) onde esso va valutato tenendo presente che anche nel decorso spontaneo, l'avviarsi a guarigione del malato di epatite virale è segnato dallo sblocco della diuresi. A conferma di un meccanismo di tipo osmotico possiamo dire che per dosi inferiori a g 1/kg fino a dosi di g 0,5/kg non abbiamo osservato né negli animali né nell'uomo un significativo incremento della diuresi.

Per quanto si riferisce, invece, all'intervento del bicarbonato di sodio, bisogna ricordare come questo sale vengano riconosciute proprietà diuretiche che sono da ascrivere allo ione carbonico (HCO_3^-) il quale normalmente viene riassorbito dal tubulo prossimale previa trasformazione in H_2CO_3; se gli H^+ necessari a tale trasformazione sono in quantità insufficiente rispetto all'eccesso di HCO_3^-, questo viene escreto come $NaHCO_3$ e trascina con sé una quantità isoosmotica di acqua onde l'aumento della diuresi (3). Ma nel nostro caso, va considerato che l'aggiunta del bicarbonato di sodio all'acido ascorbico porta a vivace effervescenza e quindi ad eliminazione di CO_2, per cui non ci pare possibile postulare l'intervento dell'HCO_3 nel meccanismo diuretico.

Unico effetto metabolico importante rilevato, è stato la diminuzione della potassiemia che, nei singoli casi, non sempre corrispondeva ad un incremento della potassiuria; si tratta però di una sperimentazione eseguita in pazienti che presentavano una potassiuria di base già aumentata e comunque ancora troppo limitata per consentire conclusioni definitive. Ci sembra possibile pertanto prospettare solo delle ipotesi. Diminuzione della potassiemia può verificarsi, a parte i casi di insufficiente assunzione o di perdita per via gastro-enterica che qui non interessano, a causa di:

a) perdita con le urine per:

1) blocco del riassorbimento del K^+ al livello del tubulo prossimale (come fanno i diuretici tiazidici): nel nostro caso, un tale meccanismo è del tutto ipotetico;

2) alcalosi metabolica indotta dall'introduzione di un sale di un acido debole ed una base forte che, imponendo il recupero a livello tubulare della massima quantità di H^+ porta, parallelamente, ad un aumento dell'escrezione di K^+;

b) ingresso del potassio nelle cellule: può soltanto accennarsene in via del tutto ipotetica, tanto più che l'alcalosi crea piuttosto le condizioni per una fouriuscita di potassio dalle cellule che non per un suo ingresso (5).

In conclusione, sia pure con le riserve dovute all'incompletezza delle nostre osservazioni, riteniamo che allo stato attuale del meccanismo più probabile della dimostrata azione diuretica dell'ascorbato di sodio sia da identificare con una diuresi osmotica.

RIASSUNTO

CAGLI V., CANTORE G. P., PECORI GIRALDI J., VIRNO M., *Azione diuretica dell'ascorbato di sodio: osservazioni sugli animali e sull'uomo.*

La somministrazione per via endovenosa nell'animale e nell'uomo del sale sodico dell'acido ascorbico alle dosi di g 1/kg di peso corporeo, ha posto in evidenza un notevole effetto diuretico.

Malgrado si tratti di dosi elevate, esse si sono rivelate assai distanti da quelle letali e, in pratica, perfettamente tollerate dall'animale e dall'uomo.

Unico effetto metabolico di rilievo conseguente alla somministrazione del farmaco è risultato essere la diminuzione della potassiemia non sempre accompagnata ad un proporzionale aumento della potassiuria.

Si prospetta, come più probabile meccanismo della diuresi indotta da questo sale, quello osmotico.

Résumé

CAGLI V., CANTORE G. P., PECORI GIRALDI J., VIRNO M., *Action diurétique du sel sodique de l'acide ascorbique.*

L'administration par voie intraveineuse du sel sodique de l'acide ascorbique à dose de g 1/kg a mis en evidence un remarquable effet diurétique chez l'animal et chez l'homme.

Les AA. ont démontré que ces doses, bien qu'elles soient élevées, s'éloignent beaucoup de doses mortelles et sont parfaitement tolérées par l'homme et par l'animal.

L'unique effet metabolique important qu'on a observé à la suite de l'administration de cette substance, a été la diminuition de la potassiemie qui n'était pas toujours accompagnée d'une proportionelle augmentation de la potassiurie.

Selon les AA., la diurèse provoquée par le sel sodique de l'acide ascorbique pourrait s'expliquer au moyen d'un mécanisme osmotique.

Summary

CAGLI V., CANTORE G. P., PECORI GIRALDI J., VIRNO M., *On the diuretic action of the ascorbic acid (sodium salt).*

The intravenous administration of ascorbic acid (sodium salt) at doses of g 1/kg of body weight was

shown to produce a marked diuretic effect in man and in the animal.

Though the doses employed were high, they proved to be very distant from lethal ones and, practically, were well tolerated by man and the animal.

The only important metabolic effect observed following the drug's administration was the decrease of kaliemia, which was not always accompanied by a proportional increase of kaliuria.

The AA. claim that an osmotic mechanism would probably account for the diuresis induced by this salt.

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(3) Goodman L. S., Gilman A., *Le basi farmacologiche della terapia*, I Ed. Italiana, Casa Editrice Dr. Francesco Vallardi, Milano, 1963.

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LA SEMEIOTICA E LA DIAGNOSI DELLE MALATTIE ACUTE IN ARETEO DI CAPPADOCIA

G. SELMI - F. RASPADORI

« Fino a Traiano quanto si sapeva di meglio a medicina, tutto si accolse, in prudentissima celta in Areteo, da Traiano ad Antonino tutto i accolse in Galeno ». Questa frase che abbiamo iportato integralmente dal Puccinotti ci dimostra l'importanza che ha avuto Areteo sullo sviuppo della medicina del periodo romano. Nato n Cappadocia, gli studiosi sono concordi nell'affermare che egli abbia vissuto in quel periodo dell'Impero romano che prese il nome dalla famiglia Flavia. Alcuni storici lo considerano tra i pneumatici, altri lo considerano a sé, come indipendente da ogni scuola esistente alla sua epoca. Si discosta dalla scuola pneumatica in più luoghi (Pazzini) come per esempio nella concezione generale della malattia che è per lui una rottura di quell'equilibrio di solidi, di liquidi, di spiriti che costituisce lo stato di salute. Oltre che la fisiopatologia, curò anche l'anatomia, facendo delle ottime descrizioni delle parti ammalate. Alcuni di queste, come per esempio quella riferita alle ulcerazioni intestinali nei dissenterici, sono talmente particolareggiate da far pensare che egli, per essere così bene a conoscenza dei processi anatomopatologici li avesse visti e di conseguenza avesse necessariamente eseguito necroscopie, cosa che non risulterebbe si facesse in epoca romana. Ma lo scopo di questa nostra nota è di rilevare la veramente notevole descrizione delle malattie che egli ci ha lasciato. I primi codici manoscritti di Areteo furono raccolti e resi noti per opera di un dottissimo medico italiano: Paolo Giulio Crasso di Padova. Molti altri ne furono scoperti nelle varie biblioteche d'Europa. Il suo libro principale dal titolo « Malattie acute e croniche » scritto in greco venne pubblicato (Pazzini) nella versione latina a Venezia nel 1552, nel testo originale greco a Parigi nel 1554, in italiano nel 1838 a cura di F. Puccinotti.

E' appunto da questa ultima pubblicazione che abbiamo attinto le nostre notizie.

Limitando il presente lavoro alle malattie acute, ricorderemo come l'Areteo, abbia descritto tutte le principali conosciute alla sua epoca.

Parlando della emottisi precisa innanzitutto che il sangue « rigettato dalla bocca » può avere due origini: può cioè derivare solamente e indirettamente dalla bocca stessa senza espettorazione ed in questo caso si parla di sputo di sangue, può al contrario derivare da parti più profonde (polmoni, bronchi, ecc.) e si deve perciò parlare di rigurgito di sangue. I sintomi che accompagnano l'emottisi, sono il dolore e la pesantezza al capo, il ronzio alle orecchie, il rossore alla faccia, il turgore delle vene e la minaccia di vertigine. Il sangue emesso non è molto denso, è scuro, leggero non permisto ad altra materia. Descrivendo la polmonite ricorda che i sintomi principali sono rappresentati da febbre acuta, con assenza di dolore se non vi è un interessamento pleurico. Il malato è affannoso, vuol star seduto, tiene eretto il tronco per meglio respirare. Gonfiore alle vene del collo, polso celere e tosse con sputo frequentemente sanguigno sono gli altri sintomi principali del quadro clinico della polmonite che solitamente porta a morte l'ammalato al 7° giorno, dopo che le estremità si sono fatte fredde e pallide ed il polso si è rimpicciolito. Con tosse accompagnata a vario escreato si presenta anche la pleurite, la quale peraltro è caratterizzata da un forte dolore che si estende fino alle spalle ed alla scapole. In questa malattia il respiro è difficile come pure l'espettorazione, vi è rossore al

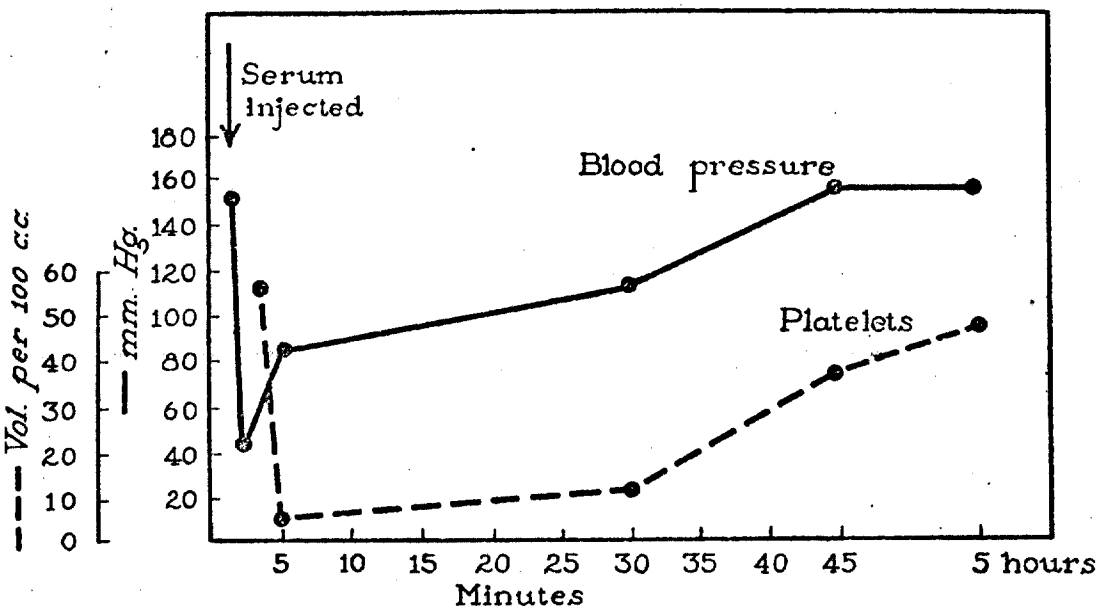

Fig. 1.
Platelets and blood pressure of a dog followed for 5 hours. As seen, 5 hours after the injection of 0.10 ml of immune rabbit serum for each kilogram of body weight the volume of platelets reached approximately the normal values.

values (Fig. 1). In addition to the reduction of platelets there was a drastic reduction of the number of leukocytes in the dog (Table III).

Summary. Serum of rabbits immune to sheep erythrocytes injected intravenously in appropriate doses in guinea pigs and dogs produced a drastic reduction of the concentration of blood platelets even though these quantities of serum provoked only moderate shock or no shock.

When the shock was deep, generally the platelets disappeared from the blood.

The injection of glycogen in doses that reduced the blood platelets in the guinea pig did not prevent the production of shock following the injection of the immune serum.

The experiments here presented show that there is not always a parallel relation between the decrease of blood platelets and the intensity of the pulmonary symptoms in the guinea pig or the decrease of arterial pressure of the dog in heterophil anaphylaxis.

15270

Subacute and Chronic Toxicities of Ascorbyl Palmitates.

O. GARTH FITZHUGH AND ARTHUR A. NELSON.

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

The fatty acid monoesters of l-ascorbic and d-isoascorbic acid prepared by Swern *et al.*[1] were suggested for use as antioxidants for fats and oils by Riemenschneider *et al.*[2] In cooperation* with these investigators we have made a study of the subacute and chronic oral toxicities of the palmitates of these acids.

[1] Swern, D., Stirton, A. J., Turer, J., and Wells, P. A., *Oil and Soap*, 1943, **20**, 224.

[2] Riemenschneider, R. W., Turer, J., Wells, P. A., and Ault, W. C., *Oil and Soap*, 1944, **21**, 47.

* The Eastern Regional Research Laboratory of

TABLE I.
Mean Gain in Weight of Male Rats Fed Diets Containing Palmitates for Six Months.

Substance	Dosage %	No. of Animals	Mean Gain Wt g	Standard Error of Mean g
l-ascorbyl palmitate	2	8	350.6	±17.1
	5	8	231.9	±22.3*
d-isoascorbyl palmitate	2	8	375.5	±14.3
	5	7	269.6	±15.1*
d-isoascorbic acid	1	10	405.3	±11.3
Control		9	394.7	±12.6

* p = <.001.

The results are reported in the following.

Experimental. For the subacute toxicity experiment 10 weanling male rats, selected at random with respect to litter mates from our colony of Osborne-Mendel strain, were placed on each of 6 diets. Ground commercial rat biscuits with 1% added cod liver oil served as the basic diet. The ascorbyl palmitates were mixed with the basic diet by means of a rotary batch mixer. Two groups of rats were placed on diets containing 2% and 5% concentrations of l-ascorbyl palmitate; two groups were placed on diets containing 2% and 5% d-isoascorbyl palmitate; one group was placed on a diet containing 1% d-isoascorbic acid and the sixth group received the control diet. Three rats were chosen from each of 6 litters and assigned to different diets as required for balanced incomplete blocks[3] of 3 with 6 treatments, where the blocks correspond to litters and treatments to diets. The design as a whole was replicated twice and thus a total of 60 rats was used. All animals were kept in individual cages in a room with controlled temperature and humidity and were given free access to their respective diets and water. Body weights and food consumption were determined at weekly intervals.

the U. S. Department of Agriculture furnished the palmitate monoesters and lard samples and partially defrayed the expenses of this study.

[3] Fisher, R. A., and Yates, F., *Statistical tables for biological, agricultural, and medical research*, Oliver and Boyd, Edinburgh, 1938.

In the chronic toxicity study 6 groups of 10 rats each were selected and assigned to their respective diets similarly to those in the subacute experiment. In addition to a 5% lard sample, with or without added palmitate, the ingredients of the diets were casein 18%, dextrose 61%, brewer's yeast 5%, salt mixture (U.S.P. XII No. 2) 4%, corn oil 3%, whole liver powder 2%, and cod liver oil 2%. The ration for each of the 6 groups contained 5% of one of the following lard samples,† namely, heat-treated lard containing 1% or 5% l-ascorbyl palmitate, heat-treated lard containing 1% or 5% d-isoascorbyl palmitate, heat-treated lard without added palmitate, and fresh lard. All diets were refrigerated at —6.7°C.

Results, Subacute Toxicity. This experiment was terminated at 36 weeks when all surviving animals were sacrificed. The data on the gains in weight during the fast growing period of the first 26 weeks (Table I) show that the animals on the 5% concentrations of the two palmitates grew significantly (p = <.001) less than those on the control diet. At the 2% levels the differences between experimental and control animals were not statistically significant (p = .05, or less, is significant for this experiment). Although the gains in weight of the animals on the 1% d-isoascorbic acid were slightly greater than those of the controls, these differences were not significant. During the next 10 weeks, however, this group grew significantly faster than the controls. Because of this fact and also because all animals of the group were living, they were continued over a 2-year experiment; at the end of 2 years the surviving animals on the d-isoascorbic acid had no gross or microscopic pathological differ-

† The palmitates were first incorporated in lard. The heat-treated lard samples with palmitates were prepared by heating fresh lard containing the palmitates at 100° C in a beaker with mechanical stirring and with free access to air for about 12 hours. The heat-treated lard without added palmitate was prepared by heating fresh lard at 100°C with washed air bubbling through the lard for 8 hours. Peroxide values showed that the heat-treated lard without the added palmitate was rancid.

ences from the control animals of other experiments and their mortality rate was what would be expected for control animals in our colony.

At 36 weeks, with the exception of the 1% d-isoascorbic acid group which showed no mortality, all groups of rats on the palmitate diets had fewer survivors than the group on the control diet. The figures for the percentage mortality were as follows: 5% d-isoascorbyl palmitate, 50%; 5% l-ascorbyl palmitate, 30%; 2% d-isoascorbyl palmitate and l-ascorbyl palmitate, 20% each; and the control 10%. The only group differing appreciably in mortality from the control, although not significantly with the small number of rats, was the 5% d-isoascorbyl group. Here there was a preponderance of deaths from acute pneumonia, but whether this should be attributed to the palmitates is doubtful.

All of the 60 rats started in the subacute series were autopsied, and 37 were microscopically sectioned. Lung, heart, liver, spleen, pancreas, stomach, small intestine, kidney, adrenal and testis were sectioned in all rats, while colon, lymph node, bone, bone marrow, thyroid and parathyroid were sectioned occasionally.

Two rats showed distinct lesions as a result of the palmitate feeding, and these were unusual. Both rats had been fed 5% l-ascorbyl palmitate for 36 weeks. Their urinary bladders were packed with numerous white mulberry stones up to 5 mm in diameter, one of which on crystallographic examination was found to consist of rods and needles of calcium oxalate monohydrate. Since oxalic acid has been reported[4] to be an oxidation product of ascorbic acid this would seem to be a reasonable source of the oxalate in the stones. In the feeding of over 50 substances to several thousand rats in 2-year chronic toxicity studies we have not observed spontaneous bladder stone formation. Stones have been produced with relative frequency by the feeding of glycols[5] and in one instance each by the feeding of barium chloride and saccharic acid. The last mentioned stone was composed of calcium oxalate, as were those caused by feeding glycols; the remaining stone was composed of barium sulphate in an organic matrix. In each rat with bladder stones one kidney and its ureter were markedly distended by the obstruction from the stones. Whitish material similar to the stones was seen in both kidney pelves of one rat. A small amount of colorless anisotropic crystalline calcareous material was seen in the parenchyma of one of the kidneys near the pelvis; this was the only kidney where such parenchymal crystalline material was seen. The kidneys in the 2 rats with urinary tract stones showed no inflammatory changes, although the bladders did show epithelial hyperplasia. Among the remaining 58 rats the surprising features were the almost total absence of calcareous material and of inflammatory changes within the kidneys and the lack of transitional examples in the gross appearance of the urinary tracts. Nor did the microscopic sections show any inflammatory changes or evidence of stone formation, with one exception, in a rat on 5% l-ascorbyl palmitate. There was a moderate degree of a focal, rather acute inflammatory process involving the interstitium and tubules of both cortex and medulla. Although this particular type of reaction has rarely been observed previously in our rats, it is not necessarily attributed to the palmitate, as unusual lesions do appear spontaneously in our rats from time to time.

Grossly, the viscera of the rats on the 5% palmitate diets, especially the l-ascorbyl palmitate, were slightly smaller than those of the controls and of the rats on 2% palmitate. Microscopically, except for the renal changes accompanying the 2 examples of stone formation and for the few spontaneous lesions that were observed, there was no outstanding difference among the 4 experimental groups and the control group. The amount of hemosiderin in the spleens of the group fed 5% palmitate was about half that in the remaining groups. The adrenals in all the experimental groups were slightly larger than those in the controls; however,

[4] Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, 1937, **117**, 237.

[5] Morris, H. J., Nelson, A. A., and Calvery, H. O., *J. Pharm. and Exp. Therap.*, 1942, **74**, 266.

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there was no microscopic difference. The testes were undamaged, with rare exceptions.

Results, Chronic Toxicity. When the gains in weight were analyzed for the experimental and control animals for the first 24 weeks and for the first year, the differences were not statistically significant for any group. During the fast growing period individual growth curves of litter mates were enough alike to be considered identical. Growth curves of the experimental groups showed no significant deviation from the control, or from each other.

Each of the 4 rats which died during the first year was from a different group, therefore, their deaths were not related to treatment. During the second year deaths were distributed over the whole period and could not be attributed directly to the effect of the palmitates. At the termination of the experiment no group had a death rate greater than that of the control.

Histopathological study similar to that of the subacute toxicity series was made of the tissues of 36 of the 60 animals and showed no outstanding gross or microscopic difference between any of the experimental groups and the control group, nor were there any distinct lesions attributable to or prevented by the palmitates. The one group of rats which did differ slightly from all the others was that fed heat-treated lard without the added palmitates. In this group, the livers usually had a slight brown or chocolate tint rarely seen in the other groups. Microscopically, lymphocytic infiltration more frequently accompanied the small amount of focal renal tubular atrophy seen equally in all the groups, testicular atrophy was greater in degree than in all other groups, and lung involvement by chronic infectious processes and by lymphosarcoma was less frequent than in the rats whose diet included lard with added palmitates or unheated lard. However, an analysis of these data from the small number of animals in this experiment does not show a statistically significant difference.

Hematological studies made at intervals during the first year of the experiment showed no toxic effect on the blood from any of the chronic dosages of the palmitates.

Summary. When l-ascorbyl palmitate and d-isoascorbyl palmitate were fed subacutely for 9 months in concentrations of 2% and 5% of the diet, only the 5% levels were toxic. The growth rates of rats on the 5% levels were significantly retarded and bladder stones were produced in 2 animals on the 5% l-ascorbyl palmitate. d-Isoascorbic acid in a concentration of 1% of the diet had no deleterious effect. Chronic toxicity studies for 2 years indicate that l-ascorbyl and d-isoascorbyl palmitate are not toxic in concentrations of 0.25% (2500 p.p.m.), or less, of the total diet of rats. Heat-treating fresh lard containing the palmitates before adding it to the diet had no effect on toxicity.

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Use of Thermocouples for Determination of the Rectal Temperature of Rats.

DAVID M. TENNENT AND MARTHA A. MELOY. (Introduced by H. Molitor).
From the Merck Institute for Therapeutic Research, Rahway, N. J.

When measurements of the rectal temperatures of rats were made in this laboratory with thermometers or with thermocouples while the animals were held in the investigator's hand, the readings were unstable and frequently changed by as much as one degree centigrade during the space of a few minutes. The results were also unsatisfactory when mechanical holders made of wire mesh were used, since the rats became unduly excited. To avoid these difficulties a method has been devised, using thermocouples, for taking the

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Effect of Reducing Agents on Copper Deficiency in the Chick [1,2]

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ABSTRACT The effect of additions of reducing agents to copper-deficient chick diets was investigated. Ascorbic acid and isoascorbic acid, at 0.1%, and dimercaptopropanol (BAL) at 0.025% of the diet decreased growth and either decreased the elastin content of the aorta or increased mortality in those groups of chicks receiving a copper-deficient, but not a copper-supplemented diet. Diphenylphenylenediamine (DPPD) had no such effect. Ascorbic acid had no effect on zinc or iron deficiency. Dietary ascorbic acid reduced the uptake of Cu^{64} by the liver whether the isotope was given orally or intraperitoneally.

Copper metabolism is influenced by a number of dietary factors other than the level of the element present. It has been found that the addition of small amounts of zinc, cadmium, and silver to a copper-deficient diet resulted in a more severe copper deficiency (1–3). Dick and Bull (4) reported that an increase in dietary molybdenum reduced the copper content of the liver. Later, Dick (5) noted that dietary sulfate was required for molybdenum to exert this influence.

Aside from these influences of known substances, there are evidently other dietary factors which influence copper metabolism. Lewis and Allcroft (6) reported that copper deficiency can occur in lambs and cattle consuming diets apparently normal in copper, molybdenum, and sulfate content. In many instances little correlation has been found between the copper content of the diet and the severity of copper deficiency symptoms when animals are pastured (6).

In an investigation of the influence of dietary factors affecting copper metabolism, attention was directed toward one of the roles associated with copper. In those oxidase enzymes containing copper, such as laccase, ceruloplasmin, and possibly cytochrome oxidase, that part of the metal component which exists in the +2 valence state undergoes reduction and subsequent re-oxidation during the course of the enzymatic reaction (7,8). This dependence upon oscillating valence states for function suggested the possibility that reducing agents might influence copper metabolism. The experiments reported herein were designed to investigate the possibility.

EXPERIMENTAL

The chicks used throughout these studies were White Plymouth Rocks obtained from a commercial hatchery. They were housed in conventional battery brooders with raised wire floors. The watering and feeding troughs were made of stainless steel. The drinking water was demineralized by passing it through an ion exchange resin column. For the zinc-deficient experiment the battery was painted with an epoxy resin paint.

The basal diet used to produce a copper or iron deficiency was based on dried skim milk and glucose as described previously (9). The diet used to produce a zinc deficiency was based on an isolated soybean protein as described by Ziegler et al. (10) except that starch instead of glucose was used.

Copper additions were made by the use of reagent grade $CuSO_4 \cdot 5H_2O$ to supply 25 ppm; iron additions by the use of reagent grade $FeSO_4 \cdot 7H_2O$ to supply 100

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ppm; zinc additions by the use of reagent grade ZnO to supply 50 ppm.

The influence of reducing agents was evaluated by the addition of ascorbic acid, isoascorbic acid, 2-3-dimercapto-1-propanol (BAL), and diphenyl-*p*-phenylenediamine. BAL was added to the diet at 0.025%, the other reducing agents at 0.1%.

In all the experiments, 2 groups of 20 chicks each were fed each diet from the day of hatching.

Elastin determinations were made on the aortas of 3-week-old chicks fed each diet in experiments 1 and 2. The method of elastin measurement and the section of the aorta used were as described previously (9).

Hemoglobin was determined as the oxyhemoglobin.

In the isotope experiment 5 μc of Cu^{64} were given either orally or intraperitoneally. At subsequent times, up to 48 hours after administration of the isotope, 5 chicks fed each diet were killed, the livers removed and weighed, and portions taken for radioactivity determination in a deep-well scintillation counter. The results, corrected for background counts and decay, are expressed as a percentage of the total dose in the whole liver.

Copper determinations on livers from chicks fed a copper-deficient diet and that diet supplemented with ascorbic acid were carried out by the use of an atomic absorption spectrometer.

RESULTS AND DISCUSSION

The results of the studies designed to assess the effects of the reducing agents, ascorbic acid, isoascorbic acid, dimercaptopropanol (BAL), and diphenylenediamine (DPPD), are presented in tables 1 and 2. Elastin determinations were made in experiments 1 and 2 but omitted in 3 and 4 because of the high correlation between mortality and reduced aortic elastin.

The presence of ascorbic acid, isoascorbic acid, or BAL resulted in decreased growth of copper-deficient chicks as well as a reduction in aortic elastin, or an increase in mortality. The ascorbic acids did not depress growth of chicks receiving the copper-supplemented diets. The effect of BAL in inhibiting growth was much less in the presence of adequate copper than in its absence. Neither aortic elastin content nor mortality was affected by these reducing agents in the presence of adequate copper. The presence of these

TABLE 1

Effect of ascorbic acid and DPPD on growth and aortic elastin of copper-deficient chicks

Supplement	3-Week wt [1]	3-Week elastin [2]
	g	*% wet wt of aorta*
Experiment 1		
None	164	8.7
Ascorbic acid, 0.1%	126	6.3
Copper, 25 ppm	242	12.1
Copper, 25 ppm + ascorbic acid, 0.1%	239	12.9
Experiment 2		
None	217	8.5
Ascorbic acid, 0.1%	164	5.5
DPPD, 0.1%	203	8.4
Copper, 25 ppm	246	13.0

[1] Mean of 2 groups of 20 chicks each.
[2] Mean elastin content of 5 chick aortas.

TABLE 2

Effect of ascorbic acid, isoascorbic acid, and BAL on growth and mortality of copper-deficient chicks

Supplement	3-Week wt [1]	3-Week mortality [2]
	g	*%*
Experiment 3		
None	159	20
Isoascorbic acid, 0.1%	120	35
Ascorbic acid, 0.1%	127	57
Copper, 25 ppm	192	15
Copper, 25 ppm + isoascorbic acid, 0.1%	205	05
Copper, 25 ppm + ascorbic acid, 0.1%	201	05
Experiment 4		
None	182	15
BAL, 0.025%	103	77
Copper, 25 ppm	219	5
Copper, 25 ppm + BAL, 0.025%	187	7.5

[1] Mean of 2 groups of 20 chicks each.

TABLE 3

Effect of ascorbic acid on the copper content of the liver

Supplement	2-Week [1] body wt	Copper content of liver [1]	
	g	*μg/g*	*μg/liver*
None	136	0.74	3.33
Ascorbic acid	110	0.73	2.99

[1] Each value the mean of 20 determinations.

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TABLE 1

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	3-Week wt [1]	3-Week elastin [2]
	g	% wet wt of aorta
Experiment 1		
	164	8.7
1%	126	6.3
	242	12.1
0.1%	239	12.9
Experiment 2		
	217	8.5
1%	164	5.5
	203	8.4
	246	13.0

oups of 20 chicks each.
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TABLE 2

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	3-Week wt [1]	3-Week mortality [2]
	g	%
Experiment 3		
	159	20
0.1%	120	35
1%	127	57
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2-Week [1] body wt	Copper content of liver [1]	
g	μg/g	μg/liver
136	0.74	3.33
110	0.73	2.99

e mean of 20 determinations.

TABLE 4

Effect of ascorbic acid on movement of Cu^{64} into the liver

Hours after isotope administration	Oral		Injected IP	
	Control	Ascorbic acid [1]	Control	Ascorbic acid [1]
	% dose in liver [2,3]	% dose in liver [2,3]	% dose in liver [2,3]	% dose in liver [2,3]
3	4.6	3.0	9.5	10.4
6	6.0	4.0	12.0	13.5
14	10.0	5.2	16.7	11.5
24	7.5	7.5	23.0	13.9
48	10.2	6.8	16.1	10.2

[1] Ascorbic acid at 0.1% in the diet.
[2] Five microcuries given orally and intraperitoneally.
[3] Each value the mean of 5 livers.

3 reducing agents, then, resulted in an increased severity of copper deficiency symptoms.

The presence of DPPD did not result in decreased growth nor decreased aortic elastin content. This reducing agent is relatively insoluble and poorly absorbed. These experiments suggest, therefore, that the effect of reducing agents in increasing the severity of copper deficiency is related to the internal metabolism of the element rather than to an inhibitory effect on absorption. Further support for such a view resulted from the observation on the copper content of the livers of chicks fed the copper-deficient diet alone and this diet supplemented with ascorbic acid (table 3). Although ascorbic acid resulted in decreased growth, the copper content of the liver was not significantly depressed either in concentration or total amount.

To obtain some insight into the possible effect of ascorbic acid on the movement of copper into the liver, radioactive copper was given both orally and intraperitoneally to the control and the ascorbic acid-fed chicks. At various times after the isotope administration the livers of 5 chicks fed each diet were examined for radioactivity. The results are presented in table 4.

The livers from chicks receiving ascorbic acid generally contained less radioactivity than those from the control chicks whether the isotope was given orally or intraperitoneally. This effect was most noticeable after 6 hours. These results suggest that dietary ascorbic acid does influence the movement of copper, either decreasing movement into the liver or increasing movement from the liver. It is possible that the effect of ascorbic acid in increasing the severity of copper deficiency is mediated by this influence on the movement of copper throughout the body.

One other point remained to be examined, the specificity of the reducing agent-copper interaction. It was possible that the effect of these agents was general and not confined to copper. To determine whether this was so, the effect of ascorbic acid on iron and zinc deficiency was examined. The results are presented in table 5. Neither of these deficiencies was enhanced by the presence of dietary ascorbic acid. This observation indicates some degree of specificity for the reducing agent-copper interaction, suggesting that

TABLE 5

Effect of ascorbic acid on zinc and iron deficiencies

Supplement	3-Week wt [1]	Hb [2]
	g	g/100 cm³
Experiment 5 (zinc deficiency)		
None	126	
Ascorbic acid, 0.1%	131	
Zinc, 50 ppm	248	
Zinc, 50 ppm + ascorbic acid, 0.1%	243	
Experiment 6 (iron deficiency)		
None	194	4.09
Ascorbic acid, 0.1%	195	4.90
Iron, 100 ppm	238	7.48
Iron, 100 ppm + ascorbic acid, 0.1%	263	7.26

[1] Mean weight of 2 groups of 20 chicks each.
[2] Mean of determinations from 10 chicks.

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it is not a generalized phenomenon associated with decreased absorption or transport of elements in general, for instance.

Whether these observations have any relation to manifestations of copper deficiency in animals raised under practical conditions, the results of these studies indicate that the presence of reducing substances in a purified diet can significantly increase the severity of copper deficiency.

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EFFECT OF ASCORBIC ACID, D-ISOASCORBIC ACID AND GLUCOASCORBIC ACID ON COBALT POLYCYTHEMIA IN THE RAT AND RABBIT

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BARRON and Barron (1) have demonstrated that the simultaneous administration of ascorbic acid with a cobalt salt to rabbits prevented the development of polycythemia. If their animals were allowed to develop the polycythemia, then the intravenous administration of 60 mg. of ascorbic acid per day resulted in a decrease in both the red cell count and the hemoglobin content. Davis (2) found that the oral administration of 8 mg/kg. of ascorbic acid to dogs reduced the polycythemia resulting from the administration of cobaltous chloride. On the other hand Chen (3) reported that supplementing the diet of mice with ascorbic acid increased the red cell count and hemoglobin concentration. No cobalt was used in his work with mice.

The present study was initiated in order to determine whether ascorbic acid had a similar effect in the polycythemic rat as that reported in the case of the dog and rabbit. Also, an attempt was made to determine whether the reported effect was due to the ascorbic acid *per se* or whether compounds with similar structures but without the Vitamin C activity could alter the course of cobalt polycythemia in the rat and the rabbit.

METHODS

Polycythemia was produced in hooded rats by the daily administration of cobaltous chloride subcutaneously at a level of 1 mg. per day, 6 days a week. This is essentially the method of Orten (4). The animals received the cobalt injections during the entire study. The diet consisted entirely of Purina dog chow. When the elevated hemoglobin concentration appeared stable for at least two weeks, the various substances to be tested were administered either orally or intravenously. The oral feedings were accomplished by the use of a rigid stomach tube. All intravenous injections were made in the tail veins. When rabbits were used the animals were rendered polycythemic by the subcutaneous administration of 10 mg. of cobalt chloride per day in the form of a 1 per cent solution (1). The rabbits were fed Purina rabbit chow supplemented by cabbage twice a week. After the animal became polycythemic the ascorbic acid and its derivatives were given intravenously into one of the marginal ear veins. All animals were still receiving the cobalt when the test acids were injected.

Hemoglobin concentrations were estimated by the acid hematin method with the final readings being made in a photoelectric colorimeter. The instrument was calibrated with human blood whose oxygen capacity had been determined.

Ascorbic, D-isoascorbic, and D-glucoascorbic acids[1] were tested for their possible effect on cobalt polycythemia. In the case of the rats, ascorbic and isoascorbic acids were given at two levels, 25 or 50 mg. per day. Glucoascorbic acid was administered at a level of 32 mg. per day, an amount equimolar to 25 mg. of ascorbic acid. Solutions to be given intravenously were neutralized with the theoretical amount of $NaHCO_3$ immediately before their administration. The rabbits all received the 50 mg. dose of ascorbic and isoascorbic acids and a 64 mg. dose of the glucoascorbic acids.

TABLE 1. EFFECT OF ASCORBIC, ISOASCORBIC, AND GLUCOASCORBIC ACIDS ON THE HEMOGLOBIN CONCENTRATION OF THE BLOOD OF THE RAT

SUPPLEMENT	DOSE	ROUTE	NO. OF ANIMALS	HEMOGLOBIN		
				Before supplement	Weeks after supplement	
					1	8
	mg/day					
Control, no cobalt	0		15	14.1 ± 0.2[1]	14.2 ± 0.3[1]	14.1 ± 0.1[1]
Control, plus cobalt	0		9	17.9 ± 0.2	17.8 ± 0.3	18.4 ± 0.2
Ascorbic acid	50	Orally	4	18.2 ± 0.7	17.8 ± 0.6	18.3 ± 0.5
Ascorbic acid	25	Orally	5	17.8 ± 0.4	17.9 ± 0.3	17.8 ± 0.6
Isoascorbic acid	50	Orally	5	17.1 ± 0.1	17.2 ± 0.7	17.5 ± 0.7
Isoascorbic acid	25	Orally	5	18.4 ± 0.3	17.0 ± 0.3	17.3 ± 0.5
Glucoascorbic acid	32	Orally	5	17.7 ± 0.4	18.1 ± 0.3	17.4 ± 0.7
Ascorbic acid as sodium salt	25	I. V.	11	18.4 ± 0.3	17.8 ± 0.3	18.1 ± 0.5
Isoascorbic acid as sodium salt	25	I. V.	6	17.8 ± 0.2	17.8 ± 0.4	17.7 ± 0.4
Glucoascorbic acid as sodium salt	32	I. V.	8	17.4 ± 0.3	17.5 ± 0.3	17.1 ± 0.4

[1] Standard error $= \pm \sqrt{\Sigma d^2/N(N-1)}$.

RESULTS

The first group of rats received orally 50 mg. per day of either free ascorbic or isoascorbic acids for a two-week period without producing any effect on the hemoglobin level of the blood. From these data it appears that neither orally administered ascorbic nor isoascorbic acids had any effect on the polycythemia produced by cobalt. The data are summarized in table 1.

In the remainder of the study on rats smaller doses of the various acids were used because attempts were made to administer the test substances on an equimolar basis, and free glucoascorbic acid possesses a limiting solubility. The oral administration of ascorbic or isoascorbic acid at 25 mg. per day for two weeks did not in-

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[1] These compounds were kindly supplied by Charles Pfizer and Company, New York.

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ds and a 64 mg. dose of the

ORBIC ACIDS ON THE HEMOGLOBIN
F RAT

	HEMOGLOBIN	
	Weeks after supplement	
	1	2
2[1]	14.2 ± 0.3[1]	14.1 ± 0.1[1]
2	17.8 ± 0.3	18.4 ± 0.2
	17.8 ± 0.6	18.3 ± 0.5
	17.9 ± 0.3	17.8 ± 0.6
	17.2 ± 0.7	17.5 ± 0.7
	17.0 ± 0.3	17.3 ± 0.5
	18.1 ± 0.3	17.4 ± 0.7
	17.8 ± 0.3	18.1 ± 0.5
	17.8 ± 0.4	17.7 ± 0.4
	1, ± 0.3	17.1 ± 0.4

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fluence the hemoglobin concentration of the blood of rats receiving cobalt. Glucoascorbic acid also was without effect when it was administered at an equimolar level. When similar concentrations of these three acids were neutralized with an equivalent amount of $NaHCO_3$ and injected intravenously in rats no effect was observed on the polycythemia during the two week test period.

In the rabbit glucoascorbic acid intravenously administered had no general effect on the polycythemia produced by cobalt. Two of the three animals tested showed a fall in hemoglobin concentration following the injection of isoascorbic acid (fig. 1).

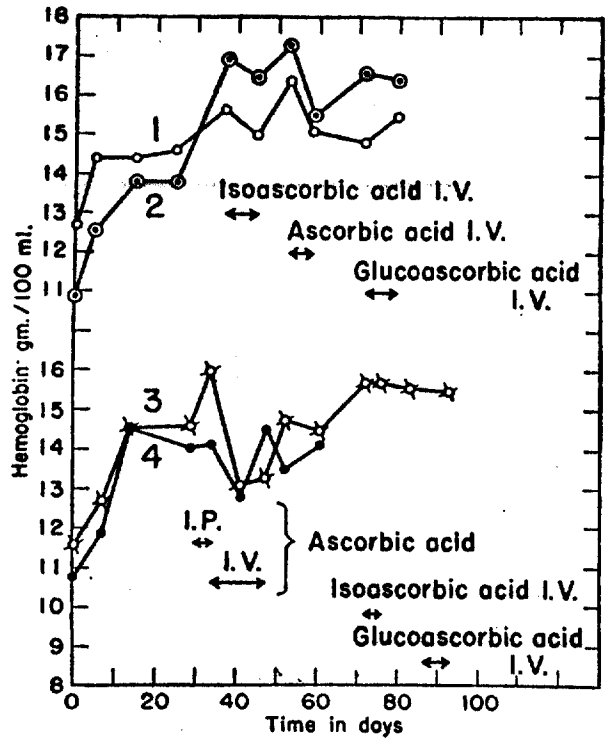

Fig. 1. EFFECT OF ASCORBIC, isoascorbic, and glucoascorbic acids on polycythemia produced in the rabbit by administration of cobalt. Numbers in the chart designate the individual rabbits used in the study.

Ascorbic acid caused a marked decrease in the hemoglobin concentration in all of the animals tested. In one rabbit, however, there was an increase in hemoglobin following the initial fall, while the animal was still receiving the ascorbic acid. It may be concluded that ascorbic acid reduces the increased hemoglobin concentration produced by cobalt in the rabbit. Isoascorbic acid also does this but to a lesser extent. This is summarized in figure 1.

DISCUSSION

From the data presented it appears that free ascorbic acid when given orally or its sodium salt when administered intravenously does not reduce the polycythemia produced by cobalt in the rat. Isoascorbic and glucoascorbic acids were also without effect on the polycythemia. On the other hand, intravenously administered ascorbic

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acid produced a marked drop in the hemoglobin concentration of rabbits made polycythemic by cobalt. Isoascorbic acid may also reduce the hemoglobin concentration in these animals but to a smaller extent. The action of the ascorbic acid in reducing the polycythemia in the rabbit is in agreement with the work of Barron and Barron (1).

From this limited study it appears that there is a parallelism between the antiscorbutic power of the compounds tested and their ability to reduce the hemoglobin concentration of polycythemic rabbits. Ascorbic acid caused the greatest drop in hemoglobin concentration and is also the most potent antiscorbutic agent. Isoascorbic acid caused a smaller drop in the hemoglobin concentration. This acid has been reported to possess only one twentieth the biological activity of ascorbic acid (5). Glucoascorbic acid did not cause any change in the cobalt induced polycythemia. The latter compound has been reported to possess only one-hundredth the antiscorbutic power of ascorbic acid (5). In fact, Woolley and Krampitz (6) have reported the production of a condition which they believed to resemble vitamin C deficiency in both cotton rats and mice by feeding glucoascorbic acid at levels of 10 per cent of the diet.

SUMMARY

Polycythemia was produced in rats and rabbits by subcutaneous daily injections of cobalt chloride. The oral administration of either ascorbic, isoascorbic or glucoascorbic acid to the rat did not influence the polycythemia. Likewise, the intravenous administration of the sodium salts of either ascorbic, isoascorbic, or glucoascorbic acid did not have any effect on the polycythemia in the rat. The intravenous administration of the sodium salt of ascorbic acid caused a marked decrease in the hemoglobin concentration of the blood of polycythemic rabbits. Isoascorbic acid, as the sodium salt, produced a similar decrease but of a smaller magnitude. Glucoascorbic acid was without effect on the polycythemia of the rabbit.

Acknowledgement is made to Dorothy L. DeZelia and Simon Kalish for aid during this work.

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The uptake of D-araboascorbic acid (D-isoascorbic acid) by guinea-pig tissues

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1. Guinea-pigs on a low, controlled intake of ascorbic acid (0·3 mg/100 g body-weight) were given daily an oral supplement of L-ascorbic acid (AA) or of D-isoascorbic acid (isoAA) (both 1·5 mg/100 g body-weight). The AA was deposited in the tissues, the isoAA was not.

2. Intramuscularly administered isoAA was deposited in the tissues but not to the same extent as equivalent amounts of intramuscularly administered AA.

3. Paper chromatography confirmed that intramuscularly administered isoAA was deposited in the tissues as isoAA, i.e. there was no conversion into AA.

4. It is concluded that in guinea-pigs (*a*) AA is absorbed from the gastro-intestinal tract much more readily than isoAA and (*b*) AA is either more readily abstracted from the blood or is more readily retained by the tissues or both, than is isoAA.

Until the biochemical systems in which vitamin C is involved have been fully characterized and reconstructed in vitro no definite statement can be made regarding the biochemical specificity of the ascorbic acids. Statements to the effect that L-ascorbic acid (L-xyloascorbic acid) (AA) has considerably greater activity than certain structurally related compounds have almost invariably been based on the results of dietary experiments and are frequently at variance with our knowledge of the behaviour of these compounds in biochemical systems. Thus D-araboascorbic acid (D-isoascorbic acid) (isoAA) is reported to have only one-twentieth of the vitamin activity of L-xyloascorbic acid, the naturally occurring isomer. This is in marked contrast to the known ability of isoAA and dehydroisoAA to replace AA and dehydroAA respectively in biochemical systems such as those involving ascorbic acid oxidase and glutathione: dehydroascorbate oxidoreductase (Dodds, 1948; Hughes, 1964; Grimble & Hughes, 1967).

Obviously 'vitamin activity' as measured by feeding experiments is an unreliable guide to the true biochemical specificity operating at the cellular level. A substance could well be judged 'inactive' on the basis of feeding trials simply because it does not reach the sites of active metabolism in sufficient quantities. Feeding experiments alone do not take into account physiological factors such as barriers to absorption, membrane selectivity and low or defective tissue retention.

Recent work has underlined this point. Hughes & Maton (1968), using the isolated erythrocyte to provide a model physiological membrane system, confirmed the finding of previous workers (e.g. Raiha, 1958) that vitamin C crosses biological membranes in the oxidized form, i.e. as dehydro-ascorbic acid. They further showed that there was virtually no uptake of dehydroisoAA (dehydro-D-araboascorbic acid) under conditions that permitted a rapid uptake of dehydroAA (dehydro-L-xyloascorbic acid). It is therefore conceivable that the apparent specificity of AA as an antiscorbutic

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agent is not so much a specificity in biochemical terms as a reflection of a selectivity imposed by certain natural membranes.

The experiments reported in this paper were designed to compare the uptake by guinea-pig tissues of similar doses of AA and isoAA.

EXPERIMENTAL

Animals and diet

Male albino guinea-pigs of initial body-weight *c.* 300 g were used. The diet had the following composition (g): ground oats 37, wheat bran 35, dried skim milk (1% fat) 10, dried full cream milk (26·5% fat) 10, dried yeast 6·5, salt mixture 1·0, and magnesium oxide 0·5. The dried milks were from Unigate (Milk Products) Ltd, the dried yeast from Distillers Co. (Yeast) Ltd, and the salt mixture from Glaxo Research Ltd. Before incorporation into the diet the milk powders were heated for 24 h at 110° to destroy any residual vitamin C. Each animal was given a weekly supplement of 0·5 ml of 5% menaphthone in arachis oil, 0·05 ml of cod-liver oil and 10 g of hay. When supplemented with L-ascorbic acid, the diet supported normal growth of guinea-pigs. Without the ascorbic acid the guinea-pigs developed scurvy.

The guinea-pigs were housed in individual galvanized zinc cages, with removable $\frac{7}{16}$ in. mesh bottoms. Pair-feeding was not practised, as previous work had indicated that moderate changes in the vitamin C intake had no appreciable effect on food intake (Evans & Hughes, 1963). The animals were weighed daily.

Ascorbic acids

L-ascorbic acid was obtained from British Drug Houses Ltd and isoascorbic acid from Koch-Light Ltd. Solutions containing the calculated amounts were prepared in glass-distilled water immediately before use, and given, intramuscularly by injection into the hind leg or orally, on a body-weight basis. The concentration of the solution was adjusted so that 0·1 ml was administered per 100 g.

AA and isoAA levels in the tissues were determined photometrically by measuring the degree of reduction of a standard acidic solution of the redox dye, phenol-indo-2:6-dichlorophenol (Bessey, 1938; Hughes, 1956). Preliminary experiments confirmed that equal weights of the two acids reduced the dye to the same extent. In experiments involving the simultaneous administration of both isomers, the 'total ascorbic acid' in the tissues (Tables 1 and 2) is therefore the sum of the isoAA and the AA.

Paper chromatography

Paper chromatography was used to establish that the isomer administered was the one actually deposited in the tissues.

The animals were killed by beheading. Their adrenal glands were quickly dissected out and ground with a little sand and 1 ml of a solution containing 0·67% metaphosphoric acid and 0·33% diamino-ethanetetra-acetic acid, sodium salt (EDTA). The extract was centrifuged and 0·005–0·010 ml of the supernatant fraction was applied to the chromatography paper; 0·01 ml of solutions of AA and of isoAA

(both containing 10 mg in 100 ml of the metaphosphoric acid–EDTA solution) were applied to each paper as standards. The papers were equilibrated in the chromatography tank for 45 min, and the isomers were separated by descending chromatography using acetonitrile:acetone:water:glacial acetic acid (80:5:15:1·2) as the solvent mixture (Kadin & Osadca, 1959). The preliminary impregnation of the paper with 2% metaphosphoric acid as suggested by Kadin & Osadca (1959) was omitted, since clearer separations were obtained with the untreated paper.

Plan of experiments

Three experiments were done.

Expt 1. Expt 1 was designed to compare the tissue levels resulting from oral dosing with the same amounts of AA and isoAA. Three groups of eight animals were used. All three groups received a daily supplement of 0·3 mg AA/100 g to satisfy normal growth requirements. Group A received no additional supplement. Group C received a further 1·5 mg AA/100 g and group B 1·5 mg isoAA/100 g. The experiment continued for 40 days. The guinea-pigs were killed and total ascorbic acid in the spleen and adrenal glands was determined.

Expt 2. Expt 2 was designed to extend the findings of Expt 1. Three groups of six animals were used. The dosage levels and distributions between the groups were as in Expt 1 but the supplements were given intramuscularly instead of orally. The experiment continued for 3 weeks. Continuation beyond this period was impracticable because of the effect of the repeated injections on the hind-leg muscles.

Expt. 3. Expt 3 was done to establish whether the isomer deposited in the tissues was the one administered, i.e. that there was no conversion of isoAA into AA, as could conceivably occur were an appropriate isomerase present. Four guinea-pigs were used. They were depleted of ascorbic acid by being given the unsupplemented scorbutogenic diet for 10 days. Two were injected intramuscularly with AA (3 mg/100 g body-weight) and two with isoAA (3 mg/100 g body-weight) daily for a period of 4 days. At the end of this period paper chromatography was used to identify the isomer deposited in the adrenals of each animal. At the same time, the concentration of the injected acid in the spleen was determined by the photometric method (see p. 212).

RESULTS

The results are summarized in Tables 1 and 2. In the guinea-pigs dosed orally (Expt 1) the group A animals (0·3 mg/100 g body-weight) had adrenal and spleen concentrations of 27·0 and 10·2 mg AA/100 g respectively. The oral intake of an additional 1·5 mg AA/100 g (group C) raised these values to 64·3 and 19·6 respectively, but the administration of a similar amount of isoAA (group B) produced no such increase. This suggests that isoAA, unlike AA, is not deposited in the tissues when given orally at a level of 1·5 mg/100 g.

The results of Expt 2 (intramuscular injection) indicate the extent to which this difference is attributable to defective gastro-intestinal absorption of isoAA. The total ascorbic acid in the adrenals of the 'isoAA' group was increased from 21·5 to 52·5 mg/100 g and in the spleen from 6·99 to 11·22. However, the tissue levels still fell short

of those obtained by injecting a corresponding amount of AA (94·0 and 31·8 mg/1 tissue for adrenals and spleen respectively). In Expt 1 there was no significant differ between the total ascorbic acid levels in the tissues of groups A and B; in Expt 2 difference was highly significant ($P < 0·01$).

The results of Expt 3 indicated that the isomer deposited in the adrenal gla was the one administered.

In the two guinea-pigs injected with isoAA the R_F values of the isomer depos in the adrenal glands were 0·39 and 0·40; in the guinea-pigs injected with AA the values were 0·36 and 0·35. The R_F values for AA and isoAA standards were 0·35 0·40 respectively.

The levels of isoAA deposited in the spleens were 8·23 and 18·01 mg/100 g. corresponding values for the guinea-pigs injected with AA were 28·50 and 3 mg/100 g respectively. These results are in agreement with the results obtaine Expt 2, the isoAA concentration being about half that resulting from a correspon injection of AA.

Table 1. Expt 1: *Effect of dosing orally with ascorbic acid (AA) and isoascorbic (isoAA) on the tissue concentrations of 'total ascorbic acid' in guinea-pig spleen adrenals. Results are given as mean values with standard errors*

	Daily supplement (mg/100 g body-wt)				Adrenals			Splee	
Group	AA	isoAA	Initial body-wt (g)	Final body-wt (g)	Weight (mg)	Wt as % of body-wt*	Total ascorbic acid (mg/100 g)*	Weight (mg)	as (r
A (6)	0·3	0	311 ± 8·31	412 ± 16·74	209 ± 10·61	0·049 ± ·002	27·0 ± 0·69	468 ± 22·45	1
B (8)	0·3	1·5	305 ± 10·39	410 ± 19·45	212 ± 16·97	0·052 ± 0·0035	26·8 ± 1·63	471 ± 38·18	
C (6)	1·8	0	308 ± 7·50	452 ± 12·25	167 ± 5·31	0·037 ± 0·0004	64·3 ± 3·18	499 ± 14·29	1

The number of animals present in each group at the end of the experiment is given in parenthe
* Difference between means of groups A and C significant at 0·1 % level.

Table 2. Expt 2: *Effect of intramuscular injection of ascorbic acid (AA) and isoasc acid (isoAA) on the tissue concentrations of 'total ascorbic acid' in guinea-pig spleen adrenals. Results are given as mean values with standard errors*

	Supplement (mg/100 g body-wt)				Adrenals		Spleen	
Group	AA	isoAA	Initial body-wt (g)	Final body-wt (g)	Weight (mg)	Total ascorbic acid (mg/100 g)*††	Weight (mg)	as (m
A (5)	0·3	0	280 ± 7·16	334 ± 18·34	128 ± 6·71	21·5 ± 1·48	523 ± 44·72	(
B (4)	0·3	1·5	272 ± 9·5	310 ± 14·5	136 ± 9·5	52·5 ± 5·6	458 ± 56	
C (5)	1·8	0	276 ± 8·05	320 ± 11·63	146 ± 8·05	94·0 ± 8·72	381 ± 13·42	

The number of animals present in each group at the end of the experiment is given in parenthe
* Difference between means of groups A and B significant at 0·1 % level.
** Difference between means of groups A and B significant at 5 % level.
† Difference between means of groups B and C significant at 0·1 % level.
†† Difference between means of groups B and C significant at 5 % level.

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standards were 0·35 an
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g from a correspondin

AA) and isoascorbic ac
in guinea-pig spleen a

	Spleen	
otal rbic acid 100 g)*	Weight (mg)	T ascor (mg
0±0·69	468±22·45	10·
8±1·63	471±38·18	8·
2±3·18	499±14·29	19·

men. given in parentheses
at 0·1 % level.

acid (AA) and isoascorb
in guinea-pig spleen a

	Spleen	
al acid g)*††	Weight (mg)	T ascor (mg
1·48	523±44·72	6·
5·6	458±56	11·
8·72	381±13·42	31·

ment is given in parentheses
nt at 0·1 % level.
nt at 5 % level.
nt at 0·1 % level.
nt at 5 % level.

DISCUSSION

The main finding to emerge from this work is that isoAA administered orally to guinea-pigs at an intake level of 1·5 mg/100 g body-weight is not deposited in the body tissues. This is in striking contrast with the behaviour of an equivalent daily dose of AA administered orally. Parenteral administration of isoAA, on the other hand, increased the level of total ascorbic acid in the adrenal glands by 144% and the level in the spleen by 61%. This suggests that the gastro-intestinal membrane(s) is not equally permeable to both isomers. This difference in permeability is probably at least in part responsible for the different tissue levels resulting from dosing orally with the two isomers.

A similar situation exists with the uptake of the oxidized forms of the two isomers by the isolated mammalian erythrocyte, and there is evidence that AA is absorbed from the gastro-intestinal tract of the guinea-pig, at least in part, in the oxidized form (Hughes & Lewis, 1965; Hughes & Maton, 1968).

The chromatographic demonstration that injected isoAA is deposited as such in the tissues indicates that there is no conversion of isoAA into AA, such as could presumably occur were the appropriate isomerase present. Also, it makes less likely the possibility that metabolic responses elicited by isoAA administration are vicarious ones resulting from a protective effect on the residual stores of AA in the tissues.

It may be noted that the levels of tissue AA in group A (0·3 mg/100g body-weight) were higher in Expt 1 (oral administration) than in Expt 2 (intramuscular injection), whereas in group C (1·8 mg/100 g body-weight) the opposite was so. At first sight these results would appear to be mutually contradictory. Two considerations should, however, be borne in mind: (*a*) oral dosing is probably more effective at low dietary intakes of AA than at high ones (Penney & Zilva, 1946), and (*b*) the continued injection of the guinea-pigs in Expt 2 probably produced stress effects. Stress in guinea-pigs lowers the level of vitamin C in the adrenals (Chatterjee, 1967) and this would presumably be more apparent at low levels of vitamin C than at high levels. Furthermore, recent work has indicated that the amount of administered AA deposited in the tissues is related to a number of factors such as sex, age and rate of growth (R. E. Hughes and P. R. Jones, unpublished observations). In Expt 2 the growth rate was less than in Expt 1, probably because of the stressing effect of the injections, and this in turn would influence the tissue deposition of the vitamin C. Such differences in the basal values for different experiments do not, however, invalidate a comparison of the levels obtained in the different groups within a single experiment.

The finding that parenteral administration of isoAA did not raise the tissue levels to those obtained with AA administered parenterally means that a factor (or factors) other than a difference in gastro-intestinal absorption is involved. Thus one can visualize a second type of 'barrier system' similar to that occurring at the absorption level but interfering with the passage of the isoAA from the blood to the 'retention areas' of the tissues. Different rates of elimination by the kidneys could also be of significance. Also of relevance is the possibility of a difference in the mechanisms of retention. Fiddick & Heath (1967) have recently shown that, in the rat adrenal, up to

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40% of the ascorbic acid is present in a 'bound form' in association with a larger molecule. Preliminary investigations indicate that in the case of the guinea-pig adrenal gland the percentage of 'bound AA' is not large (R. E. Hughes and R. J. Hurley, unpublished observations). Nevertheless, any such binding will presumably imply a specificity on the part of the 'binding' molecule; and this could be reflected as different degrees of retention of isomeric forms of ascorbic acid. Experiments in progress have been designed to cast further light on the 'binding' of ascorbic acid isomers by tissues. In the adrenal gland, where ascorbic acid appears to have certain 'extra-antiscorbutic' involvements, any such binding between AA and a larger molecule could be of considerable metabolic significance.

During the course of this work, Fabianek & Herp (1967) reported that large doses of isoAA (10 mg/day and over) could prevent the appearance of scorbutic symptoms in guinea-pigs, without producing the growth rate obtainable with much lower intakes of AA. A daily intake of 0·5 mg/100 g body-weight per day failed to support growth. This latter finding is in agreement with our results. At a daily intake level of 1·5 mg isoAA/100 g body-weight we found no tissue deposition of isoAA; nor was the adrenal hypertrophy which is characteristic of low vitamin C intakes prevented (Table 2). With the larger doses used by Fabianek & Herp (1967) presumably sufficient amounts of the isomer were absorbed to support growth.

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Nutritional Studies with Isoascorbic Acid in the Guinea Pig

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The antiscorbutic activity of isoascorbic acid in guinea pigs has been studied. As much as 250 mg. per day did not support guinea pigs fed a vitamin C–deficient diet. Administration of isoascorbic acid tended to slow down the development of acute vitamin C deficiency. In vitamin C–depleted animals isoascorbic acid had no therapeutic effect, whereas such animals responded to ascorbic acid. Animals maintained on a suboptimal intake of ascorbic acid showed some response to isoascorbic acid. These observations suggest that isoascorbic acid has a protective effect on ascorbic acid within the body, but no significant antiscorbutic activity per se.

Concurrent with the synthesis and establishment of the structure of ascorbic acid, about 25 years ago, there appeared a number of chemically related compounds. A particularly interesting compound in this group is isoascorbic acid, which has been studied by several investigators for its antiscorbutic activity. Yourga, Esselen, and Fellers (*8*) in 1944 reported isoascorbic acid as having one twentieth the antiscorbutic activity of ascorbic acid. This figure has been frequently cited in considering the nutritional relationship between the two acids. Isoascorbic acid is a potent reducing agent and has been suggested as an antioxidant in foods. The present paper describes nutritional studies of the antiscorbutic activity of isoascorbic acid in guinea pigs.

The structural formulas of ascorbic and isoascorbic acids are identical, with the exception of the configuration about the fifth carbon atom. In this presentation, we use the term isoascorbic acid for the material, *Chemical Abstracts* names it *d*-araboascorbic acid.

Methods

The primary means of determining nutritional responses is by measurement of the growth curves of young guinea pigs which is a type of study employed in many investigations similar to this one. The guinea pigs were maintained in individual cages with a supply of food and water available at all times. The scorbutic diet employed was Rockland rabbit ration. A preliminary experiment indicated that this diet was comparable to the ascorbic acid–deficient diet supplied commercially by Nutritional Biochemicals, Inc., and to the synthetic diet used by Sherman, LaMer, and Campbell (*7*). In all subsequent experiments Rockland rabbit ration was used as the deficient diet. The control diet employed to show optimal growth was freshly milled Rockland guinea pig diet (ascorbic acid fortified). In all the experiments the diet supplements were prepared fresh daily and administered by stomach tube daily. The duration of each experiment was not less than 50 days, and over 200 pigs were employed over a 12-month period. A group of animals in each instance involved five guinea pigs and, throughout this report, average weight responses for the group are indicated.

Results

Figure 1, bottom, shows the average weight response curve of five guinea pigs maintained on the deficient diet. These animals rapidly decreased in weight and by the end of four weeks all were dead. Clear indications of scurvy were observed at 3 weeks, which corresponded with the classical picture of scurvy as described by Cohen and Mendel (*1*). In Figure 1 are also shown the average weight response curve of the group maintained on the Rockland guinea pig control diet, and the average weight response curve of a group on the deficient diet plus a daily supplement of 10 mg. of ascorbic acid. The animals with the supplement show a normal weight response, indicating the adequacy of the deficient diet with respect to all nutrients except vitamin C.

The growth curves in Figure 1, third from top, compare the average weight response of animals on the deficient diet with similar groups which were given daily supplements of isoascorbic acid. A supplement of 2 mg. per day of isoascorbic acid slowed the weight loss of the group and prolonged life. However, all the pigs lost weight and died with typical signs of scurvy. The use of 10, 100, and and 250 mg. per day supplements of isoascorbic acid had a small increasing effect in slowing weight loss and prolonging the life of the animals on the deficient diet. Even with such massive doses of isoascorbic acid, typical signs of ascorbic acid deficiency developed. In contrast, supplements of ascorbic acid as small as 1 mg. per day had a considerably different effect, and as indicated in Figure 1, 10 mg. of ascorbic acid per day gave optimal growth with the deficient diet.

Figure 1, second from top, shows the effect of various amounts of isoascorbic acid supplements on animals maintained on a suboptimal intake of 0.3 mg. of ascorbic acid. This experiment was carried out, because results of the preceding study demonstrated a protective effect of isoascorbic acid, even though the compound did not prevent signs of acute ascorbic acid deprivation. The deficient diet alone resulted in rapid loss of weight. The broken line shows the weight response curve of a group of animals receiving 0.3 mg. of ascorbic acid per day. This amount of vitamin C did not provide adequate nutrition and by the end of 6 weeks all the pigs were dead. Addition of 1 mg. of isoascorbic acid had little effect, the average weight curve being somewhat lower than the curve of the animals on 0.3 mg. of ascorbic acid alone. Supplements of 10 and 100 mg. of isoascorbic acid had an influence in slowing weight loss, but were not sufficient to prevent scurvy and death.

A third type of study involved determination of the therapeutic properties of isoascorbic acid (Figure 1, top). Groups of animals were kept on the deficient diet for 2 weeks with no supplements of any kind. At the end of this time after definite weight loss had occurred, daily supplements of isoascorbic acid and ascorbic acid were started. As indicated, the groups receiving 1, 10, and 100 mg. of isoascorbic acid continued to lose weight and the animals died. A very slight difference was observed between groups on 1, 10, and 100 mg. of isoascorbic acid. Also shown are the growth curves of deficient animals given supplements of ascorbic acid following the depletion period; 0.1 mg. of ascorbic acid had no effect, whereas 1 and 10 mg. had a profound therapeutic influence.

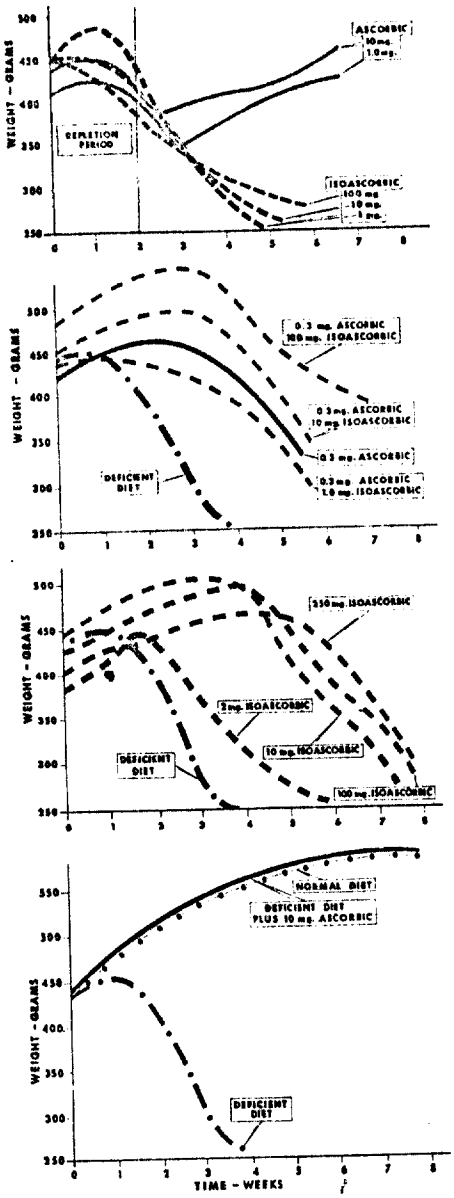

Figure 1. Weight response of guinea pigs on deficient diets

Discussion

Throughout the literature isoascorbic acid has been described as having one twentieth the antiscorbutic activity of ascorbic acid. As early as 1933, Dalmer and Moll (*2*) reported isoascorbic acid to possess one twentieth the antiscorbutic activity of ascorbic acid when given parenterally and one fortieth the antiscorbutic activity of ascorbic acid when give orally. The following year Demole (*3*) stated that he had found that the antiscorbutic activity of isoascorbic acid in guinea pigs was ". . . . about 1/20 of the activity of l-ascorbic acid, in agreement with the results of Dalmer and Moll." Also in 1934 Ohle, Erlbach, and Carls (*6*) indicated that they had supplied isoascorbic acid to von Euler, who had found that it was antiscorbutic, in agreement with Dalmer and Moll. Yourga, Esselen, and Fellers (*8*) assayed the antiscorbutic activity of isoascorbic acid using the 25-day assay procedure of Dunker, Fellers, and Esselen (*4*). They also reported that isoascorbic acid apparently had one twentieth the antiscorbutic activity of ascorbic acid.

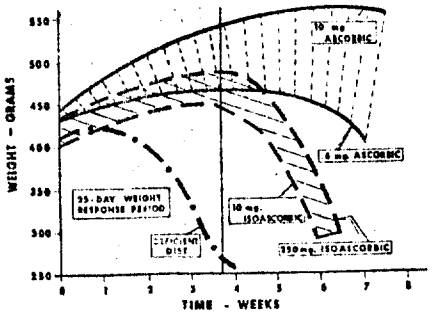

Figure 2. Composite weight response curves of animals receiving varying ascorbic acid and isoascorbic acid supplements

Shortcomings of a 25-day bioassay are emphasized by the difference in the curves in the shaded (25 days) and those observed over longer periods

New Concept of Isoascorbic Acid Activity

Only a limited amount of experimental data appears in the literature and experiments which do appear were not carried on for more than 25 days.

As Figure 2 indicates, guinea pigs supplemented with isoascorbic acid gained weight for approximately 4 weeks, but then began to lose weight and later died of scurvy. It appears that isoascorbic acid, being a potent reducing agent, protects the ascorbic acid stored in the body tissues, but, with the exhaustion of ascorbic acid reserves, it no longer produces a beneficial effect. This fact is more clearly indicated in Figure 1, top, which compares the response of a group of depleted guinea pigs receiving ascorbic acid with the response of depleted guinea pigs receiving isoascorbic acid. The two compounds have entirely different effects. One can conclude that with ascorbic acid depletion, isoascorbic acid is therapeutically ineffective. One milligram of ascorbic acid per day reversed the depletion, whereas 100 times this amount of isoascorbic acid had no beneficial effect in the depleted animals. These observations do not support the concept proposed by earlier workers (*8*) that isoascorbic acid has one twentieth the antiscorbutic activity of ascorbic acid.

From Figure 2 one can better visualize the shortcomings of a 25-day bioassay such as those appearing in the literature. The range of weight response curves for supplements of ascorbic acid ranging from 0.6 to 10 mg. per day is indicated by the area shaded with vertical dashed lines. The weight response curves for isoascorbic acid are shown by the area shaded with horizontal lines, whereas the weight response curve for the deficient diet alone is shown by the single line. During the first 25 days of supplementation, the protective period, isoascorbic acid maintains a weight increase, as does ascorbic acid. Interpretation of the weight responses during this interval could easily lead to the conclusion that isoascorbic acid has one twentieth the antiscorbutic activity exhibited by the ascorbic acid. However, if the weight curves beyond 25 days are noted, it is apparent that with isoascorbic acid weight is rapidly lost and scurvy ensues. Little difference is observed between the growth curves of 10 and 250 mg. of isoascorbic acid. Ascorbic acid has a distinctly different effect and with amounts ranging from 0.6 to 10 mg. weight increases and with the larger intake weight response coincides with that seen with an optimal diet.

Little work has been recorded on the use of isoascorbic acid in man. Ikeuchi (*5*) in Japan observed the retention of ascorbic acid and isoascorbic acid when administered to healthy subjects and vitamin C–deficient human subjects. Deficient subjects retained a large proportion of administered vitamin C, whereas a large portion of administered isoascorbic acid was excreted. These results led Ikeuchi to state ". . . its (isoascorbic acid) antiscorbutic activity in the vitamin C–deficient human body is trifling compared with that of ascorbic acid."

Isoascorbic acid has been used as an antioxidant in foods. The results of this study indicate that it may not only protect ascorbic acid in foods against oxidation, but may also protect ascorbic acid within the body.

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Differential determination of D-isoascorbic acid and L-ascorbic acid in guinea pig organs[1,2]

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To test the theory that D-isoascorbic acid (IAA) was not retained by the organs of animals, guinea pigs were fed a synthetic diet containing ascorbic acid (AA) plus IAA. The incorporation of IAA and AA was determined by making use of the different rates of osazone formation of oxidized AA and IAA with 2,4-dinitrophenylhydrazine. It was found that the organs of the guinea pigs retained a significant quantity of IAA which replaced a corresponding quantity of AA. The incorporated IAA could, in turn, be replaced by AA when only AA was subsequently given in the diet.

Introduction

D-Isoascorbic acid (IAA), also named D-araboascorbic acid and erythorbic acid, is used interchangeably with L-ascorbic acid (AA) as an antioxidant in foods and beverages with the assumption that both compounds have equal antioxidant activities, although Borenstein (3) after comparing the properties of these compounds concluded that AA was a superior antioxidant. The literature on uses of AA as an antioxidant in the food field has been thoroughly reviewed by Bauernfeind (2). Manufacturers in Canada have reported addition of both these compounds to light wines and beverages (6–100 mg %), baked goods (20–50 mg %), and meat products, cured cuts, and cured comminuted products (approximately 60 mg %), but one could expect that for economy IAA would be preferred to AA.

The antiscorbutic activity of IAA for guinea pigs has been reported to vary from zero to 1/10 that of AA (10). It has been generally believed that IAA was not retained by the body (7, 9) and that it merely spared the AA present (9). On the contrary, Fabianek and Herp (4) indicated that IAA, when given in sufficiently high doses, was able to replace vitamin C. Because of the lack of suitable methods to differentiate AA from IAA, the uptake of IAA by organs has never been ascertained.

In the present study, the incorporation of IAA by guinea pig organs and its subsequent replacement by AA were determined by an analytical procedure in which the quantitative differentiation of oxidized AA and IAA was based on their different rates of osazone formation with 2,4-dinitrophenylhydrazine (8).

Materials and Methods

Male guinea pigs weighing approximately 300–350 g were fed a synthetic diet with 0.1% vitamin C for 1 week and only animals showing satisfactory growth were used for this study. The composition of the basal diet was: vitamin-free casein, 30%; cane sugar, 20%; corn starch, 20%; corn oil, 7.4%; magnesium oxide, 0.5%; potassium acetate, 2.5%; non-nutritive fiber, 15%; salt mix (U.S.P. XIV), 4%; plus a vitamin mixture excluding ascorbic acid. The vitamin mix supplied (in grams per kilogram of ration): choline chloride, 3.5; i-inositol, 2; niacin, 0.2; p-aminobenzoic acid, 0.1; calcium pantothenate, 0.08; riboflavin, 0.03; thiamine hydrochloride, 0.02; pyridoxine hydrochloride, 0.02; folic acid, 1 mg; biotin, 1 mg; vitamin B_{12}, 0.12 mg; vitamin D, 1584 U.S.P. units; vitamin A, 9791 U.S.P. units; menadione, 2 mg; and vitamin E, 30 U.S.P. units.

To determine the uptake of IAA, eight guinea pigs were given *ad libitum* the basal diet with 0.1% AA and 2% IAA together for 1 week, while a control group of eight received the same diet with only 0.1% AA. To test the retention of IAA when followed by AA and the effect of presaturation with IAA on subsequent uptake of AA, three groups of nine guinea pigs were first depleted by being fed the basal diet devoid of vitamin C for 1 week; during the following week, 2% IAA was added to the diets of groups 1 and 2 while group 3 remained on the deficient diet; for the last week, group 2 was transferred to the diet containing 2% IAA plus 0.1% AA, and groups 1 and 3 to the diet with 0.1% AA.

The guinea pigs were anesthetized with pentobarbital and bled by heart puncture before removal of the brain, heart, liver, spleen, kidneys, and adrenals. The excised organs were immediately weighed, wrapped in Parafilm, and placed on dry ice. The frozen tissues were extracted

[1]Presented in part at the 11th annual meeting of the Canadian Federation of Biological Societies, Kingston, Ontario, June 12–14, 1968.

[2]Part of a thesis to be submitted by Omer Pelletier in partial fulfilment of the requirements for the Ph.D. degree from the Biology Department, University of Ottawa, Ottawa, Ontario.

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within the next 24 h by blending with cold 4% *meta*phosphoric acid to which 1–2 drops of antifoam FG-10 emulsion (Dow Corning) were added. Extracts were diluted to contain about 6 μg AA plus IAA per milliliter in 2% *meta*phosphoric acid, filtered, and frozen until analyzed.

The quantitative differentiation of oxidized AA and IAA was based on their different rates of osazone formation with 2,4-dinitrophenylhydrazine by the procedure for vitamin C developed in this laboratory (8).

Solutions of reagents were diluted with distilled water unless otherwise specified, and were filtered if necessary. Boric acid (5% w/v) was stored at room temperature. The following solutions were prepared every 2 weeks and kept refrigerated: stock *meta*phosphoric acid (50 g of pellets per 100 ml), 2,6-dichlorophenolindophenol (0.025% w/v), dipotassium hydrogen phosphate (45% w/v), and 2,4-dinitrophenylhydrazine (4% w/v in 9 *N* sulfuric acid). The following solutions were made fresh every day: 2% *meta*phosphoric acid (4 ml of stock solution per 100 ml), DL-homocysteine (6.25 mg/ml), thiourea – phosphoric acid (5% w/v and 64% v/v, respectively), L-ascorbic or D-isoascorbic acid (6 μg/ml in 2% *meta*phosphoric acid), and phosphoric–nitric acid mixture (2:1).

The sequence followed in the procedure is summarized in Table I. Reagents were added near the bottom of tubes having a length of about 15 cm and a diameter of about 20 mm and were mixed by vortex. Duplicate determinations were made at 32 and 52 °C. The absorbance of the final reaction product was determined at 520 mμ with a Spectronic 20 equipped with a 20-mm cuvette which could be evacuated and was rinsed with about 1 ml of the solution to be read.

TABLE I

Procedure for the determination of L-ascorbic acid and D-isoascorbic acid

Sequence	Quantity	
	Tube *a*	Tube *b*
Sample or standard	2.0 ml	2.0 ml
Homocysteine	—	0.2 ml
Boric acid	0.5 ml	—
2,6-Dichlorophenolindophenol*	Purple pink	—
Distilled water	—	As for dye
Dipotassium hydrogen phosphate	0.5 ml	0.5 ml
Homocysteine	0.2 ml	—
Room temperature	1/2 h	1/2 h
Boric acid	—	0.5 ml
Thiourea – phosphoric acid	1.0 ml	1.0 ml
2,4-Dinitrophenylhydrazine	0.5 ml	0.5 ml
Incubation (32 or 52 °C)	80 min	80 min
Cold water bath	5 min	5 min
Nitric–phosphoric acid†	5 ml	5 ml
Cold water bath	5 min	5 min
Room temperature	3/4 h	3/4 h
Absorbance (*a* vs *b*)	520 mμ	520 mμ

*The preceding steps were all done at once, and the next four steps were done for one duplicate determination before proceeding with the other.

†Nitrogen was bubbled through the solutions while the phosphoric–nitric acid was added and for 2 min afterwards.

Calculations were made as follows:

[1] μg AA/ml sample = $6\,[f(c/d)-e]/[b(c/d)-a]$

[2] μg IAA/ml sample = $6\,[e-(aq/6)]/c$

where *a*–*f* refer to absorbances: *a*, 6 μg AA at 52 °C; *b*, 6 μg AA at 32 °C; *c*, 6 μg IAA at 52 °C; *d*, 6 μg IAA at 32 °C; *e*, sample at 52 °C; *f*, sample at 32 °C; and *q* is the result obtained by Eq. [1].

AA and IAA were added to extracts of guinea pig organs and the recoveries determined on each of three days. The liver extracts were from guinea pigs depleted of vitamin C and the other organs used were from guinea pigs fed a diet containing vitamin C.

The statistical methods for evaluation of the data were those described by Snedecor (11).

Results

The time course for osazone formation from 2,4-dinitrophenylhydrazine and oxidized AA and IAA, as reflected by the increase in absorbance of osazones at 520 mμ, is illustrated in Fig. 1 (32 °C)

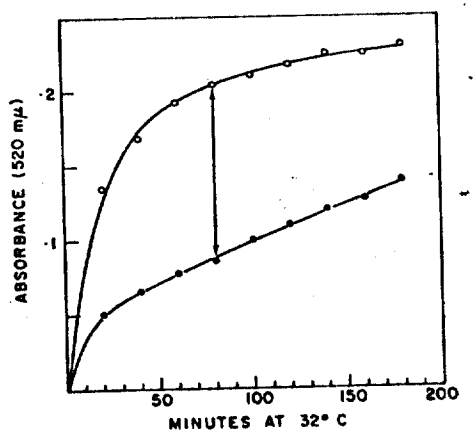

FIG. 1. Effect of incubation at 32 °C on osazone formation from L-ascorbic acid and D-isoascorbic acid (6 μg/ml). (○) Ascorbic acid; (●) isoascorbic acid.

and in Fig. 2 (52 °C). The production of osazones occurred much more rapidly from AA-containing systems than from corresponding systems containing IAA. The osazone formation with IAA was maximal after 2 h at 52 °C, but only half-completed after the same time at 32 °C. The maximal difference between the two isomers occurred after 80 min at 32 °C.

Absorbance measurements for various levels of AA and IAA at 32 and 52 °C for 80 min, as shown in Fig. 3, were linear for concentrations up to 8 μg per milliliter of assay solution.

Recoveries on three different days of mixtures of AA and IAA added to extracts of guinea pig organs are given in Table II. The values for AA were reproducible and indicated a high recovery

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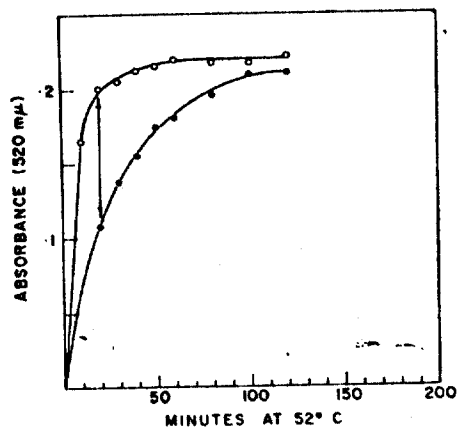

FIG. 2. Effect of incubation at 52 °C on osazone formation from L-ascorbic acid and D-isoascorbic acid (6 μg/ml). (○) Ascorbic acid; (●) isoascorbic acid.

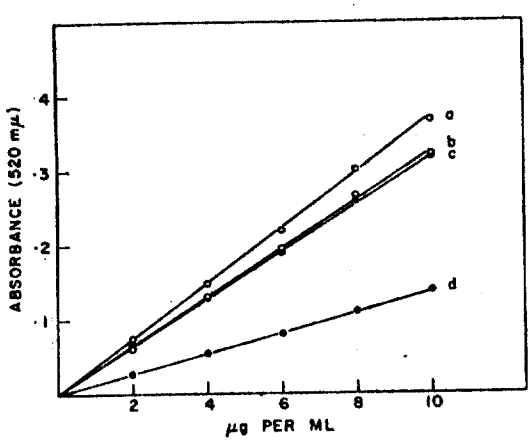

FIG. 3. Standard curves for L-ascorbic acid and D-isoascorbic acid at 32 and 52 °C. (*a*) Ascorbic acid at 52 °C; (*b*) ascorbic acid at 32 °C; (*c*) isoascorbic acid at 52 °C; and (*d*) isoascorbic acid at 32 °C.

whereas those for IAA were more variable, particularly for brain and spleen extracts.

As shown in Table III, feeding guinea pigs a diet containing mixtures of AA and IAA resulted in significant losses of AA and gains of IAA by their organs, compared with the organs of control guinea pigs receiving only AA. The total amount of AA plus IAA in the adrenals, hearts, and kidneys of the experimental animals approximated the total AA of control animals, but was less in the livers ($P < 0.05$) and spleens ($P < 0.01$). In the experimental group, the brains, spleens, and livers contained less IAA than AA, the hearts and kidneys about the same amount of each, and the adrenals more IAA than AA.

While the results reported in Table IV show a more extensive incorporation of IAA in organs of guinea pigs due to 1 week of IAA treatment preceding 1 week of AA and IAA (group 2), they also demonstrate a rapid loss of incorporated IAA during 1 week of AA treatment (group 1). In general, the total AA and IAA in the organs of these two groups approximated the total AA of the controls (group 3) to which AA was given during 1 week after the depletion period.

Discussion

The two basic requirements for the differentiation of AA and IAA were, firstly, a large difference and secondly, a minimal difference in the absorbance due to each isomer; a greater maximal difference was produced at 32 °C (80 min) than at 52 °C (20 min), but the second requirement was achieved more conveniently at 52 °C (80 min) than at 32 °C (several hours). For these

TABLE II

Typical recoveries of mixtures of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) from guinea pig organs

Organ	AA (μg ± s.d.*/ml extract)		IAA (μg ± s.d.*/ml extract)	
	Added	Recovered	Added	Recovered
Liver	1.2	1.12 ± 0.08	4.8	4.88 ± 0.17
"	1.8	1.77 ± 0.14	4.2	4.25 ± 0.20
"	2.4	2.33 ± 0.04	3.6	3.72 ± 0.10
"	3.0	2.92 ± 0.06	3.0	3.15 ± 0.09
"	3.6	3.51 ± 0.09	2.4	2.56 ± 0.12
"	4.2	4.26 ± 0.11	1.8	1.81 ± 0.17
"	4.8	4.79 ± 0.07	1.2	1.27 ± 0.13
Spleen	3.59†	3.41 ± 0.11	4.0	4.16 ± 0.70
Adrenals	2.31†	2.29 ± 0.16	4.0	4.38 ± 0.39
Kidneys	3.25†	3.31 ± 0.09	4.0	4.12 ± 0.46
Brain	7.51†	7.30 ± 0.24	4.0	3.99 ± 0.94

*Standard deviation, calculated for three assays on different days.
†Amount found prior to addition of IAA.

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TABLE III

Average uptake of D-isoascorbic acid (IAA) in guinea pigs fed a diet containing 2.0% IAA with 0.1% L-ascorbic acid (AA) for 1 week

	Experimental		Control	
Organ	IAA (mg %)*	AA (mg %)	IAA (mg %)	AA (mg %)
Liver	6.6±0.8	11.4±0.8	n.d.†	25.1±1.5
Brain	6.2±0.9	16.8±0.6	1.4±0.5	22.5±0.7
Spleen	10.1±1.8	16.7±0.8	n.d.	39.0±4.3
Adrenals	73.5±4.1	50.4±5.1	n.d.	134.8±8.3
Heart	2.8±0.5	2.0±0.3	n.d.	5.1±0.4
Kidneys	5.4±0.8	4.4±0.3	n.d.	9.6±0.5

*Mean value and standard error for eight guinea pigs.
†None detectable.

TABLE IV

Retention of L-ascorbic acid (AA) and D-isoascorbic acid (IAA) due to feeding IAA alone or mixed with AA to depleted guinea pigs

		Dietary treatments*		
Organ	Component retained†	Group 1 (IAA; AA)‡	Group 2 (IAA; AA+IAA)‡	Group 3 (Nil; AA)‡
Liver	IAA	0.5±0.4	5.8±0.4	n.d.§
	AA	14.5±2.6	6.6±0.7	15.0±1.5
Brain	IAA	2.7±1.0	10.2±1.0	0.2±0.2
	AA	15.2±2.4	8.8±0.8	17.0±1.8
Spleen	IAA	0.6±0.4	14.7±2.0	0.4±0.4
	AA	28.2±3.6	14.6±2.0	28.1±1.5
Adrenals	IAA	7.9±2.6	56.8±5.6	3.5±1.9
	AA	59.0±0.7	28.0±4.7	66.7±9.1
Heart	IAA	0.1±0.1	0.9±0.3	0.1±0.1
	AA	3.3±0.6	2.3±0.4	4.8±0.9
Kidneys	IAA	0.3±0.3	2.4±1.0	n.d.§
	AA	4.9±1.0	3.6±1.0	5.2±0.6

*Each treatment lasted 1 week; all groups were first fed the basal diet without AA or IAA; the other two treatments and their sequence were as indicated under each group.
†Mean retentions in mg % ± standard error for nine guinea pigs in groups 1 and 3, and six guinea pigs for group 2. Three animals in group 2 refused feed and were discarded.
‡AA (0.1%) and/or IAA (2.0%) were added to the basal diet.
§None detectable.

reasons, two temperatures (32 and 52 °C) at a constant time (80 min) were selected instead of only one temperature at two different times (5, 6).

The derivation of proportional equations based on reaction kinetics has been thoroughly described (5, 6), but it is possible to arrive at a similar equation by a simple rationale. Referring to Fig. 3, the following relationships are evident: $d(c/d)-c = 0$ and $b(c/d)-a = x = (b+d)(c/d)-(a+c)$. Consequently, by multiplying the 32 °C absorbance by c/d and substracting the 52 °C absorbance, the absorbances due to IAA cancel out but those due to AA do not. Similarly, the difference obtained by the expression $f(c/d) - e$ of Eq. [1] is due only to AA in the sample and $b(c/d) - a$, to AA in the standard. As shown in the numerator of Eq. [2], the absorbance due to AA at 52 °C can be calculated from the concentration q found by Eq. [1], and can be subtracted from the total absorbance at 52 °C to yield the net absorbance due to IAA.

The main advantage of the present method over previous paper chromatographic methods is that it is sensitive enough to be applied to the assay of biological specimens. Fabianek and Herp (4) had no success with two chromatographic methods for the assay of guinea pig organs. Weeks and Deutsch (13) have separated IAA from AA on silicic acid impregnated glass-fiber paper and have applied their method to foods, but since the minimum amount required is about 2 µg/20-µl spot, its application to biological specimens would be restricted to the assay of adrenals extracted in about 1 ml of solution.

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Recoveries by the present method were more accurate and reproducible for AA than for IAA. The assay values of added IAA in the presence of AA indicated that the method was suitable to determine the incorporation of IAA by the organs of guinea pigs.

The IAA assays of organs from guinea pigs receiving no IAA gave negative values in most cases and only insignificant traces in the others. The results obtained with guinea pigs fed IAA demonstrated that IAA was taken up by organs where it replaced a corresponding amount of AA. The IAA retained could in turn be replaced by AA and turned over at least as rapidly as AA, for which a half-life of 3–4 days has been reported.

It has been generally believed that IAA was not retained by tissues and that it exerted its antiscorbutic activity by sparing the AA present. When IAA was given to human subjects, depleted (10) or on intakes of 10 and 25 mg AA per day (12), a greater proportion of IAA was excreted than when the same amount of AA was given in the same way; furthermore, daily doses of IAA to depleted subjects did not increase levels in leukocytes and plasma, while AA did (10). Since the methods used by those workers did not differentiate AA from IAA, their results do not rule out a possible displacement of AA, and AA replacement by IAA.

Kadin and Osadca (7), using a paper chromatographic method which could differentiate the two isomers at concentrations above physiological levels, saturated humans with AA, and on the day after discontinuing saturation doses gave 300 mg of IAA. When they found no difference in AA excreted (during the following 4 h) as compared to control studies where IAA was omitted, they concluded that IAA had no displacement action. One could explain the uptake of IAA by guinea pigs in the present study by postulating a rapid turnover of AA with replacement by IAA. Since the turnover of AA in humans is known to be about four times longer than in guinea pigs, one would expect little turnover (about 3 mg) during 4 h in the human, with a rate of utilization of about 20 mg AA per day (1); and consequently, one would expect even less replacement by IAA. Such a small displacement would be hardly detectable in the urine excreted during 4 h. The incorporation of IAA into humans, although much less rapid, would probably be more stable than in guinea pigs.

Several authors have reported the antiscorbutic activity of IAA to be $\frac{1}{10}-\frac{1}{20}$ that of AA (12). Reiff and Free (9) using a Rockland rabbit ration devoid of vitamin C reported that daily supplements of 250 mg IAA did not support growth of guinea pigs but tended only to slow down development of acute scurvy. Since 0.3 mg AA plus 100 mg IAA could maintain growth for more than 3 weeks, but 0.3 mg AA alone could not, they concluded that IAA had a sparing effect. Recently, Fabianek and Herp (4) have demonstrated that administration of only 10 mg IAA with a deficient diet to guinea pigs previously depleted for 1 week prevented scurvy for as long as 115 days. Although they were unable to determine the nature of the "ascorbic acid" present in organs after the administration of IAA, they interpreted their results as indicating that IAA could replace vitamin C. Since the demonstration of IAA incorporation by the present study supported this hypothesis, the antiscorbutic activity of IAA with a Rockland rabbit ration and the synthetic diet described above is presently being investigated. It remains to be seen whether IAA can replace AA as a cofactor for several reactions (1).

Acknowledgments

The author thanks T. K. Murray (Nutrition Research Division, Food and Drug Directorate, Ottawa) and C. Godin (Department of Biology, University of Ottawa) for their encouragement and very useful discussions. The technical assistance of Eva Nagy and C. Desloges is gratefully acknowledged. D-Isoascorbic acid was graciously supplied by Hoffman–LaRoche Inc., Montreal.

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Kilander's Health Knowledge Test Form AM at the completion of the high school education in selected Minnesota Public High Schools.

Procedures

It was recommended that 20 schools from each of the three populations teaching health education under scheduling Plans I, II, and III would be a representative sample. Permission was obtained to administer a health knowledge test to senior pupils in the 60 randomly selected public high schools in Minnesota.

The Otis score card for evaluating standardized tests was used to select the best health knowledge tests. A curricular analysis was then made of standardized health knowledge tests developed by Bridges, Dearborn, and Kilander using the Minnesota State Course of Study as the criterion measure to compare closeness of fit on test items and sections of the course of study. The Minnesota State Course of Study covered 97 per cent of the test items in the Kilander Health Knowledge Test Form AM. This test was superior to the other two in coverage in six of the 11 sections.

The Kilander Health Knowledge Test Form AM was used to test 2,785 pupils; 689 pupils who received direct instruction under Plan I in 20 schools; 1,201 pupils who received direct instruction combined with physical education under Plan II in 20 schools; and 895 pupils who received indirect instruction in related subject areas under Plan III in 20 schools.

The test administrators administered the tests in their schools under standardized conditions between April 15 and May 15, 1956. The completed tests were returned to the investigator for scoring. The mean score for each of the 60 randomly selected schools was calculated and recorded under Plans I, II, or III as the data to be evaluated.

The basic statistical technique utilized to compare the three sets of school means was the analysis of variance. The F-test, the chi square test, and the T-test were also utilized in analyzing the data. The five per cent level of confidence was accepted as the level which was indicative of significance.

Conclusions

The following conclusions seem to have implications for the three health instructional plans:

1. Direct instruction in the nature of a concentrated course for a period of one year was found to be the significantly superior instructional plan.
2. Indirect health instruction in related subject fields was found to be the least effective plan.
3. The health instructional plans from most to least effective were found to be: (a) direct instruction in a concentrated one-year course; (b) direct instruction alternating with physical education based on a definite number of periods per week; and (c) indirect instruction in related subject areas.
4. Teacher preparation did not appear to influence the superiority of Plan I over Plans II and III.

Recommendations

The following recommendations are presented:

1. It is recommended that senior high schools of Minnesota, as well as high schools of other states where a plan of direct health instruction is not offered, should include a course in direct health instruction of at least one year in length in their curricula.
2. It is recommended that health teachers, local and state school health education supervisors, and school administrators who are presently providing health instruction in related subject areas or direct instruction in combination with physical education should incorporate a direct health instruction plan of one year's duration at the senior high school level to replace existing health scheduling plans.
3. Indirect instruction in related subject areas should be encouraged in the appropriate related subject areas. Microfilm $2.50; Xerox $5.40. 110 pages.

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HEALTH SCIENCES, NUTRITION

COMPARATIVE METABOLIC RESPONSE TO ERYTHORBIC ACID AND ASCORBIC ACID BY THE HUMAN

(L. C. Card No. Mic 60-5464)

Marian M. Y. Wang, Ph.D.
The Pennsylvania State University, 1960

The physiological response of human subjects to oral intakes of the isomers, erythorbic and L-ascorbic acids, was compared. The criteria were urinary return and plasma level of total ascorbic acid. Two load tests and a balance study were conducted.

Test loads of 165 mg and 250 mg were administered to six and five subjects, respectively, in the two load test studies. Blood plasma increments of total ascorbic acid were similar following either L-ascorbic acid or erythorbic acid test doses. Comparable plasma levels for the two forms of intake for three hours following ingestion were found for each load test study. In the balance study, six young male college students were maintained 14 days on a 25 mg ascorbic acid diet and four 12-day experimental periods on a 10 mg ascorbic acid diet. During four consecutive experimental periods, the two isomers were fed alternately as 100 mg supplements to a 10 mg ascorbic acid diet. A cross-over design of supplementation was used. Fasting blood samples were taken and 24-hour urinary collections were made on the last four days of each experimental period. With L-ascorbic acid supplementation, the mean fasting plasma total ascorbic acid level was 1.03 mg%; the mean urinary return 15.3 mg per day. For these same subjects on erythorbic acid supplementation, the mean fasting plasma level was 0.62 mg%, but the urinary returns were increased almost five-fold, 72.2 mg per day.

Erythorbic acid has little or no antiscorbutic activity. This may be because of preferential peripheral uptake of the vitamin or high excretion of erythorbic acid due to selective tubular reabsorption of the vitamin. An experiment was carried out to study the peripheral uptake and excretion rate of the two isomers.

Six male adult subjects were fed a 25 mg supplement to a 10 mg ascorbic acid diet during three 7-day experimental periods. Prior to the experimental period there was one week of adjustment, and two weeks of exploratory work when all the subjects had a daily dietary ascorbic acid e of 10 mg. On the 5th day of each experimental d, a test dose of 300 mg erythorbic acid was given to the subjects; on the 7th day a test dose of the same amount of L-ascorbic acid was given.

The peripheral uptake was measured by determining the difference between the ascorbic acid levels in arterial and venous blood. Fasting capillary and venous blood samples were taken on the test days. The fasting sample was followed by a second sampling of capillary and venous blood after the ingestion of the test dose. No increase of arterio-venous difference occurred during the elevation of plasma total ascorbic acid on either form of test dose. The arterio-venous difference method did not differentiate between the peripheral uptake of the two isomers, but it revealed a small sustained L-ascorbic acid uptake.

Urinary collection was started after the ingestion of the test dose. The 24-hour collections divided into three periods were 32%, 12.6% and 9% of the erythorbic acid test dose; and 1.7%, 1.4% and 2.2% for the L-ascorbic acid test dose. Most of the erythorbic acid was excreted within the first six hours. The high and rapid erythorbic acid excretion suggests no appreciable reabsorption by the tubular cells. Microfilm $2.50; Xerox $4.00. 75 pages.

HEALTH SCIENCES, PHARMACY

INTERACTION OF PHARMACEUTICALS WITH NONIONIC MACROMOLECULES.

(L. C. Card No. Mic 60-5637)

Sayed Sami Ahsan, Ph.D.
State University of Iowa, 1960

Chairman: Associate Professor Seymour M. Blaug

A solubility study has shown a definite complex formation between Tweens and o-hydroxybenzoic acid, p-hydroxybenzoic acid, acetylsalicylic acid and phenobarbital. No interaction was observed between the Tweens and boric acid and m-hydroxybenzoic acid. Among the hydroxybenzoic acids, the ortho isomer exhibited the strongest complexing tendency followed by the para and meta isomers. Since the molecular weights of the Tweens studied varied between a narrow range (1236-1328), the difference in complexing tendencies of the various Tweens was not highly significant. The extent to which aspirin, phenobarbital, o-hydroxybenzoic acid and p-hydroxybenzoic acid interacted with the Tweens was much greater than their reported interactions with polyethylene glycols 4000 and 6000.

Cloud point titration technique was employed to study the interaction of Tweens with phenol and resorcinol. Tweens, even in low concentrations, reacted with phenol and resorcinol to give water insoluble oily liquids.

The interaction of methyl, ethyl, propyl and butyl parabens with Tween 80, Myrj 52, Pluronic F-68 and polyethylene glycols 4000 and 6000 was studied. The general binding tendency which the parabens exhibited increased with the molecular weight of the paraben. In other words, the strongest complexing tendency was exhibited by butyl paraben followed by propyl, ethyl and methyl parabens. The hydrophil-lipophil balance of the nonionic macromolecule strongly influenced the binding tendency which the parabens exhibited for the macromolecules. The extent of complex formation in solutions of strongly hydrophilic macromolecules such as polyethylene glycols 4000 and 6000 was relatively very small when compared to the binding affinity exhibited by the polyoxyethylene macromolecules, Tween 80 and Myrj 52.

The binding of preservatives by nonionic macromolecules reduces the amount of free preservative available to protect a pharmaceutical formulation against deterioration. Data presented enables one to calculate the amount of preservative that would be required in the presence of various nonionic macromolecules to insure the presence of sufficient unbound or free preservative.

The interaction of sorbic acid with various nonionic macromolecules was investigated. The effect of Tween 80 on the preservative action of sorbic acid was also studied to determine if any correlation exists between the degree of interaction and preservative concentration required to completely inhibit the growth of Pseudomonas aeruginosa and Aspergillus niger. The complexing tendency of sorbic acid for Tween 80 was found to be less than that of the parabens hence there should be less interference with the activity of sorbic acid in formulations containing Tween 80 or similar surfactants than in formulations preserved with the parabens. A correlation was shown to exist between the predicted and experimentally determined effect of Tween 80 on the inhibitory concentration of sorbic acid in vitro.

Microfilm $2.50; Xerox $4.40. 81 pages.

THE EFFECTS OF SPRAY DRYING ON SOME PHARMACEUTICAL GUMS

(L. C. Card No. Mic 60-5134)

Richard Allen Hill, Ph.D.
The University of Florida, 1960

Four water-soluble gums of pharmaceutical importance - acacia, tragacanth, karaya and sodium alginate - were selected for preparation in spray-dried form. The resulting modification in particle size and shape was expected to produce a gum particle with the high dispersion rate characteristic of spray-dried products. This increase in rate would be an advantage in prescription compounding and manufacturing procedures provided it was obtained without adversely affecting other valuable gum properties. The investigation undertaken involved two phases: first, the preparation of the gums in a spray-dried form; and second, a comparison of the product with control samples.

Spray drying of all gums was accomplished in a Bowen Pilot Plant Spray Dryer operated at drying temperatures varying from 150 to 260° C. Homogeneous, aqueous gum dispersions of suitable concentrations were gravity fed to a centrifugal sprayer which atomized the material into a stream of hot flue gases. The dry product was removed

The items following each abstract are: the number of manuscript pages in the dissertation and its cost on microfilm. Enlargements 5-1/2 x 8-1/2 inches, 4-1/2 cents per page.

1938 Voprosy pitaniya (Problems of Nutrition) No.2

Antiscorbutic Activity of the Synthetic Sodium Salt of Isoascorbic Acid

E. M. Bamdas, B. A. Lavrov, V. M. Rodionov, N.S. Yarusova

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Received 17.XII.1937 (December 17,1937)

In the preceding, preliminary paper of one of us (1) there was shown the vitamin activity of synthetic isoascorbic acid (inthe form of its sedium salt). Here we are reporting data concerned with the vitamin C activity of this substance, which have been obtained under standard conditions of biological experimentation.

The sodium salt was prepared (Bamdas, Rodienov (2)), basically, in accordance with the Ole method from a methyl ether of 2-keto-d-gluconic acid which, in turn, was prepared synthetically in our laboratory (2, 3).

The scheme for the synthesis of the salt, in the given case, was as follows: the ether was dissolved in a doubled amount of water at 70 - 80 deg, and over the course of two hours, sodium bicarbonate was added to the solution. Following filtration and a subsequent cooling off of the solution to 0 deg, there precipitates a crystalline residue of the sodium salt of isoascorbic acid (A); from a mother liquor, that has been boiled down to half in vacuum, there precipitates still another small amount of salt (B). The residue, A, in accordance with iodine, contains 98.14% salt, and the residue, B -- 98.85%.

Biological Experiments (Therapeutic method)

The therapeutic method, which we had adopted at the State Centrol Vitamin Station, consists in that young animals, which had not disclosed, in their growth rate, any deviations from the norm, under overall nutritional conditions, are transferred to a scorbutic feed (good hay, autoclaved, oats and water) and scurvy is allowed to develop. On the 18th day of this period (in the event that a rapid drop in weight has taken place -- 1 - 2 days

earlier), the animals are subjected to therapy by means of planned doses of the substance under study, and after that, they are put to death on the 22nd day of the therapeutic period forthe purpose of histological study. A certain amount of the animals in which scurvy, from the very beginning, assumed a pronounced form, succumbed to spontaneous death before the three-week period, even in the case of large doses, and in these cases, they naturally cannot be taken into account in the experiment, inasmuch as these animals, evidently, possess a particularly increased sensitivity to scurvy. It is established, by means of histological study, through which doses recovery took place from scurvy, and in the case of which ones it did not. The minimal therapeutic dose corresponds to a biological unit of activity on the part of the substance under study. The method isbbased on a fact, strongly established in our laboratory, that in guinea pigs, close to the 10th day for a scorbutogenic procedure, a clearly indicated scurvy develops.

Experiments with a sample, A, of the sodium salt of isoascorbic acid. The experiments were set up on 4 groups of guinea pigs with doses of 15, 20, 25 and 30 milligrams, and with the number of animals being 8 in each group, during a period from theend of April to the beginning of June, 1937.

The results of the tests are brought together in Table 1.

Experiments with a sample, B, of the sodium salt of isoascorbic acid. Experiments were set up on 3 groups of quina pigs with doses of 15, 20 and 25 milligrams, with the number of animals being 7, 8 and 9, respectively, in each group, during a period from the end of April to the middle of June 1937.

The results of the tests are brought together in Table 2.

Table 1

No.of the Animal	Spontaneous Death	Put to Death	Histological Diagnosis
	days of curative period		
		Group I (dose of 15 milligrams)	
222	-	22	No scurvy
241	-	21	Scurvy not eliminated on the ribs
176	6	-	Scurvy of the II degree
258	-	22	No scurvy
242	-	22	" "
194	-	21	Scurvy of the II degree
244	11	-	" " III "
167	11	-	" " II "
		Group II (dose of 20 milligrams)	
233	-	22	No scurvy
234	-	22	Scurvy of the I degree
216	-	22	No scurvy
207	-	22	" "
217	-	22	Scurvy of the I degree ?
243	17	-	" " II "
195	-	22	No scurvy
193	19	-	Scurvy of the I degree
		Group III (dose of 25 milligrams)	
218	-	22	No scurvy
213	-	22	" "
174	-	22	" "
236	-	22	" "
215	-	22	" "
224	-	22	No scurvy ?
272	-	-	Scurvy of the II degree
225	-	22	No scurvy
		Group IV (dose of 30 milligrams)	
283	-	22	No scurvy
246	-	22	" "
264	-	22	" "
285	-	22	" "
287	-	22	" "
291	-	22	" "
253	-	22	" "
180	-	22	" "

Table 2

No. of the Animal	Spontaneous Death	Put to death Death	Histological Diagnosis
	Days of Curative Period		
		Group I (dose of 15 milligrams)	
280	-	23	No scurvy
232	-	23	Scurvy of the I degree
248	-	22	" " II "
257	-	22	" " I "
279	18	-	" " I "
261	9	-	" " II "
252	12	-	" " III"
		Group II (dose of 20 milligrams)	
221	-	22	Scurvy not entirely eliminated (ribs)
274	-	22	No scurvy
263	-	22	" "
247	-	22	" "
229	-	22	Scurvy of the I degree
197	-	22	" " I "
228	10	-	" " III"
279	7	-	" " III"
		Group III (dose of 25 milligrams)	
206	-	22	No scurvy
259	-	22	" "
245	-	22	" "
230	11	-	Scurvy of the II degree
143	5	-	" " II "
273	-	22	No scurvy
238	-	22	" "
231	-	22	" "
249	-	22	" "

Note (Guinea) Pigs Nos. 228 and 278 were transferred to therapy because of a marked drop in weight.

The results set forth in the tables indicate that a dose of 15 milligrams of the sodium salt of isoascorbic acid does not manifest any therapeutic effect. On the contrary, doses of 25 and 30 milligrams may be considered to be fully curative. A dose of 20 milligrams lies on the threshold of curative action, and in groups with this dose, we have the most variegated picture.

The minimal, therapeutic dose, thus, lies between 20 and 25 milligrams of this preparation, and, probably, close to the first dose; consequently, a biological unit is more than 20 and less than 25 milligrams, so that 1 gram of the sodium salt of isoascorbic acid contains no less than 40 biological units, more accurately, more than 40 and less than 50 units, or more than 2 and less than 2½ human doses.

By making a corresponding conversion to pure isoascorbic acid (1 gram of the sodium salt corresponds to 0.889 gram of acid), it is possible to consider that 1 gram of isoascorbic acid has an activity of close to 2.5 human doses. If one is to consider further that 1 human dose of ascorbic acid corresponds to 20 milligrams and, consequently, 1 gram of it contains 50 human doses, then isoascorbic acid, according to the data obtained, is 20 times less active than the ascorbic one; this is in complete agreement with the previously accepted calculations.

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(Translated by Carl Demrick Associates,Inc/LB/t)

ОБ АНТИСКОРБУТИЧЕСКОЙ АКТИВНОСТИ СИНТЕТИЧЕСКОЙ НАТРИЕВОЙ СОЛИ ИЗОАСКОРБИНОВОЙ КИСЛОТЫ

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Поступила 17.XII.1937

В предыдущей ориентировочной работе одного из нас (1) была показана витаминная активность синтетической изоаскорбиновой кислоты (в виде ее натронной соли). Здесь мы сообщаем данные об С-витаминной активности этого вещества, полученные при стандартных условиях биологического опыта.

Натриевая соль была приготовлена [Бамдас, Родионов (2)] в основном по способу Оле из метилового эфира 2-кето-d-глюконовой кислоты, который в свою очередь был приготовлен синтетически в нашей же лаборатории (2, 3).

Схема синтеза соли в данном случае была такова: эфир растворялся в удвоенном количестве воды при 70—80° и в течение двух часов к раствору прибавлялся бикарбонат натрия. После фильтрования и последующего охлаждения раствора до 0° выпадает кристаллический осадок натриевой соли изоаскорбиновой кислоты (А), из маточника же, упаренного до половины в вакууме, выпадает еще некоторое количество соли (Б). Осадок А по иоду содержит 98,14% соли, осадок Б — 98,85%.

Биологические опыты (терапевтический метод)

Терапевтический метод, принятый у нас на Государственной контрольной витаминной станции, состоит в том, что молодых животных, не обнаруживших в скорости своего роста отклонений от нормы на общем пищевом режиме, переводят на скорбутический корм (хорошее сено, автоклавированное, овес и вода) и дают развиться цынге. На 18-й день этого периода (в случае наступления быстрого падения веса— раньше на 1—2 дня) животные подвергаются терапии намеченными дозами испытуемого вещества и затем убиваются на 22-й день терапевтического периода для гистологического исследования. Некоторое количество животных, у которых цынга с самого начала принимает резкую форму, дает спонтанную смерть раньше трехнедельного срока даже на больших дозах, и в этих случаях их собственно нельзя засчитывать в опыт, так как такие животные, очевидно, обладают особо повышенной чувствительностью к цынге. Гистологическим исследованием устанавливается, на каких дозах произошло исцеление от цынги и на каких нет. Минимально терапевтическая доза соответствует биологической единице активности испытуемого вещества. Метод основан на строго установленном в нашей лаборатории факте, что у морских свинок около 10-го дня скорбутогенного режима всегда развивается ясно обозначающаяся цынга.

Ascor 150

Опыты с образчиком А натриевой соли изоаскорбиновой кислоты. Опыты были поставлены на 4 группах морских свинок с дозами 15, 20, 25 и 30 мг и с числом животных по 8 в каждой группе в период от конца апреля до начала июня 1937 г. Результаты испытаний сведены в табл. I.

Таблица 1

№ животного	Спонтанная смерть	Убиты	Гистологический диагноз
	дни лечебного периода		
		Группа I (доза 15 мг)	
222	—	22	Цынги нет
241	—	21	На ребрах не ликвидированная цынга
176	6	—	Цынга II степени
258	—	22	Цынги нет
242	—	22	» »
194	—	21	Цынга II степени
244	11	—	» III »
167	11	—	» II »
		Группа II (доза 20 мг)	
233	—	22	Цынги нет
234	—	22	Цынга I степени
216	—	22	Цынги нет
207	—	22	» »
217	—	22	Цынга I степени?
243	17	—	» II »
195	—	22	Цынги нет
193	19	—	Цынга I степени
		Группа III (доза 25 мг)	
218	—	22	Цынги нет
213	—	22	» »
174	—	22	» »
236	—	22	» »
215	—	22	» »
224	—	22	Цынги нет?
272	7	—	Цынга II степени
225	—	22	Цынги нет
		Группа IV (доза 30 мг)	
283	—	22	Цынги нет
246	—	22	» »
264	—	22	» »
285	—	22	» »
287	—	22	» »
291	—	22	» »
253	—	22	» »
180	—	22	» »

Опыты с образчиком Б натриевой соли изоаскорбиновой кислоты. Опыты были поставлены на 3 группах морских свинок с дозами 15, 20 и 25 мг с числом животных 7, 8 и 9 соответственно в каждой группе в период от конца апреля до половины июня 1937 г.

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Результаты испытаний сведены в табл. 2.

Таблица 2

№ животного	Спонтанная смерть	Убиты	Гистологический диагноз
	дни лечебного периода		
		Группа I (доза 15 мг)	
280	—	23	Цынги нет
232	—	23	Цынга I степени
248	—	22	» II »
257	—	22	» I »
279	18	—	» I »
261	9	—	» II »
252	12	—	» III »
		Группа II (доза 20 мг)	
221	—	22	Не вполне ликвидированная цынга (ребра)
274	—	22	Цынги нет
263	—	22	» »
247	—	22	» »
229	—	22	Цынга I степени
197	—	22	» I »
228	10	—	» III »
278	7	—	» III »
		Группа III (доза 25 мг)	
206	—	22	Цынги нет
259	—	22	» »
245	—	22	» »
230	11	—	Цынга II степени
143	5	—	» II »
273	—	22	Цынги нет
238	—	22	» »
231	—	22	» »
249	—	22	» »

Примечание. Свинки №№ 228 и 278 переведены на терапию при сильном падении веса.

Результаты, представленные на таблицах, показывают, что доза в 15 мг натриевой соли изоаскорбиновой кислоты не оказывает терапевтического эффекта. Наоборот, дозы в 25 и 30 мг могут считаться вполне куративными. Доза в 20 мг лежит на пороге куративного действия и в группах с этой дозой мы имеем наиболее пеструю картину.

Минимально терапевтическая доза, таким образом, лежит между 20 и 25 мг этого препарата и, вероятно, ближе к первой дозе, следовательно, биологическая единица более 20 и менее 25 мг, так что 1 г натриевой соли изоаскорбиновой кислоты содержит не менее 40 биологических единиц, точнее более 40 и менее 50 единиц или более 2 и менее 2½ человекодозы.

Делая соответствующий пересчет на чистую изоаскорбиновую кислоту (1 г натриевой соли соответствует 0,889 г кислоты), можно считать, что 1 г изоаскорбиновой кислоты имеет активность около 2,5 человекодозы. Если далее считать, что 1 человекодоза аскорбиновой кислоты соответствует 20 мг и, следовательно, 1 г ее содержит 50 человекодоз, то изоаскорбиновая кислота, по полученным данным, в 20 раз менее активна, чем аскорбиновая, что вполне согласуется с ранее принимавшимися расчетами.

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ON THE ANTISCORBUTIC ACTIVITY OF THE SYNTHETIC SODIUM SALT OF ISOASCORBIC ACID

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Two curative experiments were carried out on guinea-pigs in order to secure an exact estimation of the antiscorbutic activity of the sodium salt of isoascorbic acid, prepared synthetically in this laboratory.

It appears from the experimental results that the minimum curative dose amounts to 20—25 mg of this preparation, or else 1 g of the salt contains more than 40 and less than 50 biological units (i. e., more than 2 and less than $2^1/_2$ human doses).

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Antiscorbutic Activity of D-Araboascorbic Acid.* (32120)

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In 1933-36, Dalmer and Moll(1), Zilva(2) and Reichstein and Demole(3) recognized D-araboascorbic acid as a factor with antiscorbutic properties, although considerably less active than L-ascorbic acid.† Recent reports claimed that D-araboascorbic acid was not able to replace the antiscorbutic role of L-ascorbic acid(4,5). In view of the controversy concerning the vitamin activity of D-araboascorbic acid, we investigated this problem on guinea pigs fed a scorbutigenic diet supplemented with various doses of D-araboascorbic acid.

Methods and results. Experiments were performed on young adult male animals, 4-5 months-old, weighing 330 ± 40 g. The guinea pigs were initially fed Wayne Guinea Pig diet containing 40 mg L-ascorbic acid per 100 g for 2 weeks. After this adaptation period the animals were given our purified scorbutigenic diet(6,7) for one week (preparatory period) and thus depleted of ascorbic acid. The animals were then divided into 10 groups of seven each. For 38 days, 6 groups were fed the scorbutigenic diet supplemented with daily oral doses of 1, 2, 10, 50, 100 or 200 mg D-araboscorbic acid. However, 3 guinea pigs from the group that received 10 mg D-araboascorbic acid were kept in the experiment for 115 days. Three groups were fed the scorbutigenic diet and daily doses of 1, 2 or 15 mg L-ascorbic acid for 38 or 115 days. Animals of one group were fed the scorbutigenic diet exclusively until they died. In the above studies L-ascorbic acid and D-araboascorbic acid were dissolved in water and administered in volumes of 1 ml by pipet.

During the experimental period we observed the appearance of animals and recorded their weights regularly (Fig. 1 and 2); at autopsy

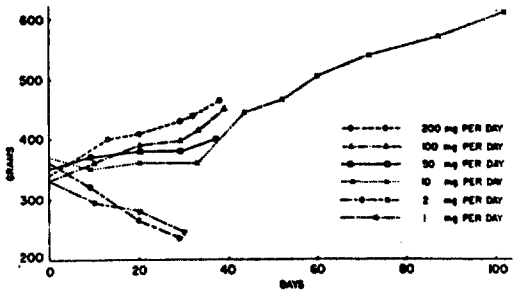

FIG. 1. Average body weight of guinea pigs receiving D-araboascorbic acid.

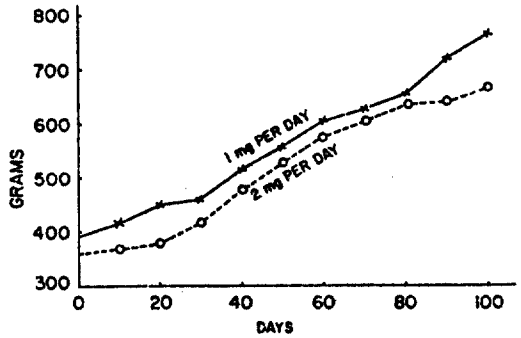

FIG. 2. Average body weight of guinea pigs receiving L-ascorbic acid.

* This work was partly aided by Nat. Inst. of Arthritis Metabol. Dis., Grant AM-4619.

† L-ascorbic acid or ascorbic acid is also known as L-xyloascorbic acid or vitamin C. D-araboascorbic acid (known also as isoascorbic acid or erythorbic acid) differs from L-ascorbic acid only in the specific configuration of — H and — OH groups around the fifth carbon.

TABLE I. He

Animal (No.)
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4*
5
6
7

* Dead on
† Dead on

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ceiving L-ascorb
acid were sacrif
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determined in th
our adaptation(8
drazine method
respectively, L-a
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drazine in the
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tensity obtained

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mg D-araboasco
appearance and
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the scorbutigeni

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TABLE I. Hemorrhages Found at Autopsy of Guinea Pigs Receiving Oral Daily Doses of 1 Mg or 2 Mg D-Araboascorbic Acid.

Animal (No.)	Hemorrhages (localization and degree)			
	Articulations			
	Chondrocostal	Tibio-femoral	Intestines	Other organs
	Guinea pigs receiving 1 mg D-araboascorbic acid a day			
1	+	++	+	0
2	+	++	++	Hypertrophy of blood vessels (+++)
3	++	+++	+	Hypertrophy of blood vessels (++)
4	+	+	0	0
5	+	±	0	0
6	+	0	0	Hypertrophy of blood vessels (+++)
7†	0	0	+	0
	Guinea pigs receiving 2 mg D-araboascorbic acid a day			
1	0	+++	+++	0
2	0	0	0	Skin (petechiae) (++)
3	0	0	0	0
4*	0	0	0	Hypertrophy of blood vessels (++)
5	+	++	+	0
6	0	0	0	0
7	0	++	+	0

* Dead on 20th day.
† Dead on 28th day.

we examined them for signs of scurvy. In some animals the ascorbic acid content of various organs was determined. Animals receiving L-ascorbic acid or D-araboascorbic acid were sacrificed 24 hours after administration of the last dose.

The concentration of "ascorbic acids" was determined in the adrenals, spleen and liver by our adaptation(8) of the 2,4-dinitrophenylhydrazine method of Roe and Kuether(9) using, respectively, L-ascorbic and D-araboascorbic acids as standards. In our preliminary studies the oxidized form of D-araboascorbic acid was found to react with 2,4-dinitrophenylhydrazine in the same way as the L-dehydroascorbic acid, but the "osazone" dissolved in sulfuric acid gave one third the color intensity obtained with L-dehydroascorbic acid.

All animals, except for the guinea pigs deprived of ascorbic acid and those on the scorbutigenic diet supplemented with 1 or 2 mg D-araboascorbic acid, presented a normal appearance and at autopsy showed no signs of scurvy or other diseases. The animals fed the scorbutigenic diet died between the 23rd and 31st days. Their average weight loss was 40% of the initial weight; they showed the typical signs of scurvy at autopsy(10,11). The guinea pigs receiving 1 or 2 mg of D-araboascorbic acid either died in 3 to 4 weeks (Table I) or were sacrificed *in extremis* on the 33rd day of the experiment. At that time, they had lost an average of 35% of their original weight. Their autopsy record is given in Table I.

In the case of guinea pigs receiving 10 mg of D-araboascorbic acid per day the "ascorbic acid" was stored in the tissues at approximately 20% lower level than that observed in guinea pigs given 15 mg of L-ascorbic acid (Table II). It was previously shown that the concentration of L-ascorbic acid in guinea pig tissues is proportional to the logarithm of the oral dose(8,12). This does not seem to apply to D-araboascorbic acid, since the concentration of "ascorbic acid" found in the tissues of animals fed 100 mg D-araboascorbic acid was only slightly higher than in those fed 10 mg (Table II). The method of analysis, however, does not reveal

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TABLE II. Tissue Concentrations of "Ascorbic Acid" of Male Guinea Pigs Treated for 38 Days with Various Doses of L-Ascorbic Acid or D-Araboascorbic Acid.

Animals receiving:	"Ascorbic acid" (mg/100 g wet organ) found in: Liver	Spleen	Adrenals
L-ascorbic acid			
1 mg per day	.98 ± .1*	6.3 ± .7	8.6 ± 1.0
2 " " "	2.6 ± .5	10.4 ± 2.0	15.4 ± 2.1
15 " " "	5.4 ± .4	18.9 ± .2	61.3 ± 3.5
D-araboascorbic acid			
10 mg per day	2.7 ± .4	17.0 ± .7	49.1 ± 4.7
100 " " "	3.3 ± .6	19.6 ± .3	57.3 ± 2.9

Each group contained 4 male guinea pigs.

For determination of tissue "ascorbic acid" L-ascorbic acid and D-araboascorbic acid were used as standard, respectively.
* Standard deviation.

the nature of the stereoisomer of ascorbic acid stored in the organs of guinea pigs treated with D-araboascorbic acid. This problem is under study.‡

Discussion. It was previously shown that L-ascorbic acid disappears rapidly from the tissues of guinea pigs. After 8 days of deprivation the organs contain 1/8th the concentration of L-ascorbic acid found in control animals fed 20 mg of this vitamin per day(12, 15,16). Therefore, at the end of the one week in which the scorbutigenic diet was not supplemented with any form of ascorbic acid, the animals became practically depleted of this vitamin. We can hence attribute the normal survival of guinea pigs receiving 10 mg or more of D-araboascorbic acid per day to the antiscorbutic activity of this compound. Thus, after 115 days, we still found complete absence of hemorrhages at autopsy in guinea pigs given daily 10 mg D-araboascorbic acid. The rate of growth of guinea pigs receiving 10 mg D-araboascorbic acid was still below that observed for the groups fed 1 or 2 mg L-ascorbic acid (Fig. 1 and 2). Indeed, for the period of 115 days, the average daily gain by the animals given 10 mg D-araboascorbic acid was 2.2 g against 2.8 g for guinea pigs receiving 2 mg L-ascorbic acid.

Dalmer *et al*(1) found that D-araboascorbic acid administered orally was 40 times less active than L-ascorbic acid. These authors giving the two isomers for 4 weeks at insufficient dose to prevent scorbutic lesions, reached their conclusions by comparing the intensities of hemorrhages at autopsy in animals receiving daily 1.5 ml lemon juice (0.5 mg L-ascorbic acid) to the hemorrhages in animals fed the sodium salt of D-araboascorbic acid (20 mg per day, orally).

However, according to more recent work, D-araboascorbic acid has no antiscorbutic activity but exerts merely a protective effect on the residual stores of L-ascorbic acid in the tissues of humans(5) or guinea pigs(4). On the contrary, the present study indicates that D-araboascorbic acid when given in sufficiently high dose, is able to replace vitamin C.

Since one of the main functions of vitamin C is its participation in collagen synthesis (17), hydroxyproline contents were determined in the 0.154 M, 0.5 M NaCl, 0.5 M citrate (pH 3.6) extracts as well as on total skin of guinea pigs fed the scorbutigenic diet supplemented with oral doses of 10 mg D-araboascorbic acid for 115 days. These analyses showed that the levels of soluble and insoluble collagen fractions and of total hydroxyproline of dermal connective tissue in such guinea pigs were the same as those found in skin tissues of guinea pigs which

‡ So far we have been unable to determine the nature of ascorbic acid stored in the organs of guinea pigs treated with D-araboascorbic acid. Two methods, namely the paper chromatographic method of Miki *et al*(13) and the thin layer procedure of Brenner *et al*(14) gave satisfactory result with pure solutions of ascorbic acids, but no yield when carried out on tissue extracts. According to personal information from the authors, the methods were never tested on animal or vegetable tissue extracts.

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Summary. D-Araboascorbic acid administered to guinea pigs in daily oral doses of 10 to 200 mg apparently replaced the antiscorbutic activity of L-ascorbic acid. The animals survived normally and the D-araboascorbic acid prevented them from developing any sign of scurvy discernible at autopsy.

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Immunity Against Experimental Cholera. (32121)

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Although cholera vaccines have been used since before the turn of the century, scientifically controlled field trials have only recently proven that they have any value in prophylaxis against cholera(1,2). The results of the field trials have been disappointing in that the vaccines which were sufficiently innocuous for wide scale usage in man conferred only a low degree of immunity of limited duration. However, one important fact emerged from these efforts: it is feasible, by means of parenteral immunization, to confer at least some degree of resistance against this bacterial infection which is restricted to the lumen of the intestine.

In previous studies on pathogenesis and immunity in experimental cholera(3,4,5), we have described a product, choleragen, which is elaborated *in vitro* under certain defined conditions by certain strains of cholera vibrios. This antigenic protein moiety, administered orally in microgram amounts with buffer, causes choleraic diarrhea in infant rabbits; it causes marked outpouring of fluid (enterosorption(6)) into the lumen of isolated loops of adult rabbit small bowel *in vivo;* in submicrogram amounts, it is an extremely potent permeability factor eliciting a delayed-sustained reaction in rabbit skin(7); and, it has been shown to cause a cholera-like syndrome in man(8). Our previous studies have shown that choleragen elicits the production of specific antibody which neutralizes and precipitates it(9). However, in our attempts to protect infant rabbits passively by parenteral administration of choleragen antiserum prior to challenge, little, if any, evidence of protection was obtained. The present communication presents evidence that parenteral administration of choleragen in adult rabbits gives rise to a highly effective degree of active immunity against intestinal challenge with either choleragen or a massive dose of living cholera vibrios. Some resistance can also be passively transferred to infant rabbits by means of serum from the immunized animals.

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FOOD ANTIOXIDANTS

Biochemistry of Erythorbic Acid. Human Blood Levels and Urinary Excretion of Ascorbic and Erythorbic Acids

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Recent widespread use of erythorbic acid as an antioxidant in foods raises the question of masking of true L-ascorbic acid levels in assessment of nutriture. The usual methods for determining ascorbic acid in blood do not distinguish between these acids. Comparative blood level trials in five normal subjects showed that the ingestion of erythorbic acid which has less than 1% of the vitamin C activity of L-ascorbic in animals, can lead to overestimation of the state of vitamin C nutrition as judged by blood levels. A rapid, quantitative paper chromatographic technique for separation of the isomers showed no significant displacement of L-ascorbic by 300-mg. doses of erythorbic acid.

Erythorbic acid, also known as D-araboascorbic or D-isoascorbic acid, a stereoisomer of L-ascorbic acid (Figure 1), has been widely used in foods in recent years as the antioxidant properties of this more easily synthesized isomer are similar (*2*) to those of L-ascorbic acid. However, marked differences have been reported in the chemical, physical, and biological properties of the two isomers as summarized in Table I. In guinea pigs, erythorbic acid was claimed to be only about 5% as potent as L-ascorbic acid with respect to antiscorbutic activity (*14*) and to effects on serum phosphatase (*3*) and excretion of intermediary tyrosine metabolites (*11*). This 1 to 20 relationship does not hold for the marked differences in reactivity of the isomers with ascorbic acid oxidases (*7*) and reductases (*1, 13*).

Recent guinea pig tests (*9*) have shown the activity of erythorbic acid to be due to its sparing action on L-ascorbic acid. In severely depleted animals, erythorbic acid has less than 1% of the antiscorbutic activity of L-ascorbic acid. Attempts to demonstrate antagonism between the isomers were unsuccessful in serum phosphatase tests in guinea pigs (*3*), but complete inhibition by erythorbic acid of an ascorbic acid oxidation enzyme in a fungus has been demonstrated (*7*). Ikeuchi (*5*) reported urinary excretion of erythorbic and ascorbic acid after dosage to vitamin C-deficient humans,

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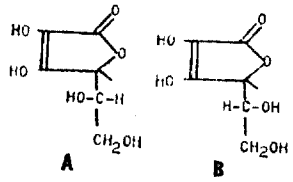

Figure 1. Stereochemical configurations

A. L-Ascorbic acid (vitamin C)
B. Erythorbic acid

which indicated little, if any, biochemical utilization of erythorbic acid.

As blood levels are normally used in assessing the state of a person's vitamin C nutrition and as commonly used clinical methods for determining blood levels of ascorbic acid will also measure erythorbic acid, the appearance of significant amounts of the latter in the blood following ingestion of this isomer would give a distorted picture of the status of an individual with regard to vitamin C nutriture. Hence, blood levels of erythorbic acid after oral dosage to humans were studied.

In addition, measurements were made of urinary excretions of L-ascorbic acid after oral doses of erythorbic acid to determine whether this less active isomer could displace L-ascorbic acid from the tissues of saturated subjects. This part of the study required an investigation of established methods for separating ascorbic from erythorbic acid by paper chromatography. A procedure was developed for quantitative colorimetric estimation of the two isomers in urine.

Experimental

Blood Studies. Blood analyses were done by the micromethod of Lowry and coworkers (*6*), a procedure commonly used for nutrition surveys. In this procedure, cupric ion converts the isomers to their dehydro forms which are then coupled with 2,4-dinitrophenylhydrazine at 37° C. followed by colorization of the coupled forms with 65% sulfuric acid. Minor deviations from this procedure include the use of whole blood instead of serum and performing the cupric ion conversion and dinitrophenylhydrazine coupling in 16 hours at room temperature instead of in 4 hours at 37° C. Use of whole blood eliminates the necessity for the considerable manipulation of blood collecting tubes and involves direct transfer of the blood to test tubes containing the protein precipitant. Furthermore, whole blood levels are considered a better index of vitamin C nutrition than serum or plasma levels (

Urinary Studies. Urinary excretion studies to test the possibility of displacement of L-ascorbic acid by erythorbic acid were performed on subjects saturated with L-ascorbic acid. To ensure saturation, the subjects were dosed orally with 500 mg. of L-ascorbic acid per day for a period of at least 7 days. Tests were run only on those subjects who, the day before, had excreted at least 40% of the 500-mg. saturating dose in the 4 hours following dosage. The 4-hour basal excretions were measured by omitting the daily 500-mg. saturation dose on that particular day. Saturation doses were again given orally until the day before the following urine collection period. Urinary levels of L-ascorbic and erythorbic acids were measured in a parallel 4-hour period after oral dosage of 300 mg. of erythrobic acid given either as a single dose or in 50- to 100-mg. increments at 15- to 30-minute intervals. Saturation doses were given through the day just preceding the test day with the time schedule exactly as followed for the basal excretion period.

Table I. Physical, Chemical, and Biological Differences

	L-Ascorbic Acid	Erythorbic Acid
Specific rotation	+20.5° to 21.5°	−16.7° to −1[illegible]
Melting point	190–192° C.	164–168° C.
Nicotinic acid addition compound	+	−
Uranium complex in alkaline solution	Unstable	Stable
Antiscorbutic activity in guinea pigs	1	0 to 1/20
Effect of serum phosphatase in guinea pigs	1	1/20
Preventing excretion of intermediate tyrosine metabolites in guinea pigs	1	1/20
Rate of oxidation by ascorbic acid oxidases	1	0 to 2/25
Rate of reduction of dehydro forms by reductase systems	1	1/3 to 1/2

Paper chromatographic separations of ascorbic and erythorbic acids in urine were made by a modification of the qualitative method of Mitchell and Patterson (*8*). The modifications, which were designed to make the separation quantitative, more rapid and applicable to urine, included use of 3-hour descending chromatography instead of the 6-hour ascending technique and prior impregnation of the paper with 2% instead of 1% metaphosphoric acid to stabilize the isomers during chromatography. The higher concentration of metaphosphoric acid is necessary to give a successful separation when a relatively large volume of urine is applied per spot. The 2% solution is prepared on the day of use. The best separation of the isomers was obtained by application per spot of 20 to 40 λ containing 10 to 20 γ of each acid. Spotting and chromatographic development in daylight led to losses of 13 to 17%, which were eliminated by spotting under Wratten OA safelights and developing the chromatograms in an unlighted room with the shades drawn.

Quantitation was accomplished by using a guide chromatogram alongside three test chromatograms, the whole consisting of four spots applied evenly along a 6 × 22½ inch sheet of Whatman No. 1 paper. After being spotted, the paper was placed in a 12 × 24 inch cylindrical chromatography jar containing about 50 ml. of the mobile solvent. This consists of a mixture of acetonitrile, acetone, water, and glacial acetic acid in proportions by volume of 80:5:15:1.2, respectively. About 1.5 hours were allowed for saturation of the sealed system before the mobile solvent mixture was added to the chromatographic troughs. After development, during which the solvent fronts approached to within an inch of the bottom, the papers were removed and dried for about 15 minutes in a ventilated hood.

The guide chromatogram was then separated by cutting lengthwise and dipped in a 0.03% aqueous solution of 2,6-dichlorophenolindophenol. White spots against a pink background indicate the exact location of the isomers. Dipping the guide chromatogram, instead of spraying as recommended in the methods investigated for separation of these isomers (*1, 8*), yielded sharper delineation of spots. The 0.03% concentration of dye is suitable for the amounts of reducing isomers in the present tests. If more or less ascorbic or erythorbic acid is present, the dye concentration can be varied from 0.05% to 0.005% as needed. The corresponding areas on the test chromatograms were cut out transversely.

As part of the reagent blanks, strips of paper of similar area were cut out below the erythorbic acid and above the ascorbic acid spots. Separate estimates of L-ascorbic and erythorbic acids and their respective blanks were made by thorough agitation of the folded paper strips in aqueous metaphosphoric acid with a rod followed by addition of the colorimetric reagents to the same vessel without removing the disintegrated strips.

Colorimetry was by the indophenol-xylene extraction technique (*10*). While the R_f values of the two isomers are fairly close, 0.38 and 0.43 for L-ascorbic and erythorbic acids, respectively, separation was surprisingly successful starting with an oval spot as large as ¼ × ½ inch on which as much as 100 λ (0.1 ml.) of urine had been applied slowly while drying with warm nitrogen. The use of these large spots eliminated the necessity for a preparative isolation of the isomers by paper chromatography

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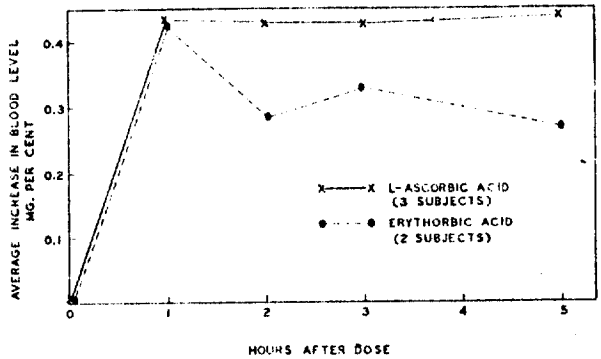

Figure 2. Whole blood levels of L-ascorbic or erythorbic acid in humans after a 500-mg. oral dose

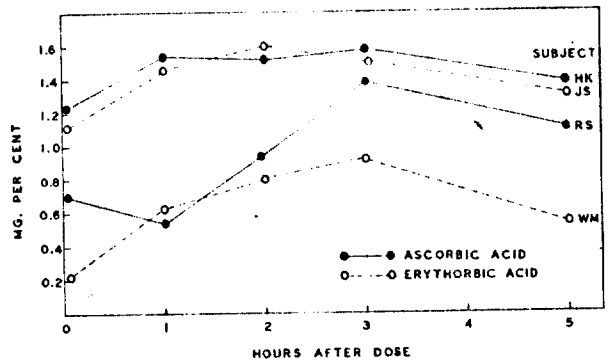

Figure 3. Average increase of whole blood levels of L-ascorbic or erythorbic acid in ascorbic acid-saturated humans after a 200-mg. oral dose

followed by a tedious and time-consuming extraction and concentration prior to a second stage paper chromatographic separation as practiced for urine and other biological extracts by Chen, Isherwood, and Mapson (*1*).

Little or no interference from other reducing substances was indicated by recoveries of 101 and 108%, respectively, for L-ascorbic and erythorbic acids added to a representative urine specimen and chromatographed by this procedure. A ratio of isomers of 5 to 1 was easily separable. Four urine specimens can be processed in an 8-hour period. The relatively short handling period minimizes losses due to the well-known instability of these isomers.

Results and Discussion

Following oral dosage to humans at the 500-mg. level, the blood level curves (Figure 2) with the two isomers are closely comparable. Subjects H. K. and J. S., whose predosage levels indicated saturation, show an almost identical rate of rise and fall on 500-mg. dosage of L-ascorbic and erythorbic acid, respectively. Subject W. M., having a basal level apparently in the deficiency range, responds to erythorbic acid dosage so that his blood levels are elevated to the vitamin C-sufficient level. As in this first experiment the doses were large and some of the subjects apparently not saturated, for the second experiment the subjects were saturated with L-ascorbic acid and the dosage was lowered to 200 mg. (Figure 3). The increases above the predose levels are closely comparable for both isomers and are maintained for at least 5 hours. Ingestion of foods to which erythorbic acid is added simply as an antioxidant could lead to an overestimation via blood level tests of the state of vitamin C nutrition.

The results of the urinary excretion tests in Table II indicate no significant displacement of L-ascorbic acid by the 300-mg. dosage of erythorbic acid. The average excretion of L-ascorbic acid is practically identical to the average basal excretion. Zilva (*15*) has reported that a slight deposition of erythorbic acid—consistent with its antiscorbutic activity—occurs in the tissues of vitamin C-deficient guinea pigs. If erythorbic acid had significant antiscorbutic activity in humans, this isomer would be expected to be deposited in human tissues and thus displace L-ascorbic acid from saturated subjects. As the present study indicates that there is no significant displacement of L-ascorbic acid by erythorbic acid, it suggests different metabolic pathways for the two isomers and appears to confirm Ikeuchi's conclusion (*5*) that the antiscorbutic activity of erythorbic acid in humans is trifling compared to that of L-ascorbic acid.

Table II. Effect of 300-Mg. Oral Dose of Erythorbic Acid on Human Urinary Excretion of L-Ascorbic Acid

Subject	Excretion in 4 Hr. after Dose, Mg. Basal	Post dosage
H.K.	27	22
W.M.	40	43
M.O.	38	59
J.S.	27	31
J.V.	26	25
	Av. 31.6	32.0

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Effects of vitamin C and related substances on the glycogen yield

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I. Introduction

That vitamin C, the known antiscurvy substance, also has an effect on the carbohydrate metabolism, has already been indicated by many authors. Stepp, Schroeder and Altenburger (1) and Sticesco and Gingold (2) found in healthy individuals a reduction of the blood sugar after intravenous application of l-ascorbic acid. Alterburger (3) observed in scurvitic as well as in healthy guinea pigs a higher glucose yield in the liver by the application of glucose with cebione than with sugar alone. Hirsch (4) could also show that cebione alone can cause an increase of the liver glycogen in healthy animals. Watanabe (5) verified in this clinic on healthy rabbits the findings by Altenburger, and he also found a re-formation of the glycogen supply, lost by liver damages, by the application of l-ascorbic acid.

But we know little at present about the nature of the vitamin C effect on the glycogen yield. The question is what is the relation between the glycogen effect of vitamin C and the antiscurvy effect. We thus have to clarify whether the former is limited only to the antiscurvy substances, or whether it occurs more widely beyond the closely related substances, which are not always characterized as antiscurvy substances. Some vegetable substances are highly effective against scurvy, as known, and the question arises whether these substances actually go parallel with the glycogen effect, and of whether the latter effect goes parallel with the vitamin C content. In order to be able to answer all these questions, I have also considered in this study, in addition to l-ascorbic acid, the known synthetic vitamin C substance, d-araboascorbic acid, which is an isomer of the former but which is said to be far inferior in its anti-

scurvy effect according to investigations by Dalmer and Moll (6), Demole (7) and Zilva (8). In addition, various vegetable substances were available in this study about which very little is known as far as their effect on the sugar metabolism. As far as I know, Collazo (9) is the only one in the literature who indicates that glucose with orange juice, introduced into the stomach of healthy rabbits, causes a higher glycogen content of the liver than glucose alone. As it will be seen from the results of the following investigations, the vegetable substances have as a rule a pronounced effect on the glycogen yield. The effects of the natural products are generally difficult to analyze, however, because of their complicated compositions. For this reason I have also used for comparison some chemically prepared flavone compounds whish were made available to me by the courtesy of Prof. Huzise of the Chemical Institute of this University, for which I want to thank him at this point.

The flavone compounds are closely related to vitamin P, as it results from the investigation by Szent-Gyoergyi (10), who isolated from lemon juice citrin, a flavone compound of hesperidin and eryodictyol glycoside, which, when introduced together with ascorbic acid, is said to prevent the development of scurvy.

II. Testing Method

The test rabbits were fed as a rule 7 days or longer with a laboratory diet which is very common here and which consisted of exxlusively of okara.

For the determination of the amount of glycogen formed in the liver it is very useful to wash out first the existing glycogen completely from the organ without excessively harming, however, the parenchyma, and to use to this end among others the lecithin method worked out by Nagao and Otomo (11), which consists in that 10 cc 10% lecithin suspension are injected intraveneously into the rabbits so that the glycogen completely

disappeared within 6 to 24 hours. In view of this fact, I always made a lecithin infusion exactly according to the above described instructions 12 hours before vitamin C or closely related substances were introduced, whose effect on the glycogen formation was to be tested, in order to get a clear picture of the newly formed glycogen yield which is not blurred by the preformed substance. In order to cause the formation of new glycogen, I introduced per kg 10 cc 25 g/l glucose solution together with the vitamin, likewise intravenously, which is said to produce a considerable amount of glycogen in the liver 3 hours later, according to Nagao and Otomo (11, 12) despite the preceding lecithin infusion.

The glycogen was determined according to the Bierry (13)-Yamakawa (14) method modified at this clinic, where the Bertrand method was used to test the reduction.

For the determination of the vitamin C content of the natural products to be tested I used the Birch-Harris-Ray-microdetermination method (17) modified by Fujita and Iwatake (16), where 2,6-dichlorophenol indophenol, which was first introduced by Tillmans (18), was always used. That the value thus found is in good agreement with the anti-scurvy effect and must therefore be considered as vitamin C content, was already verified by Harris and Ray (19).

That the glycogen content of the rabbit liver shows a varying behavior depending on the season, particularly with the supply of glucose, was studied by Kimura (20) at this clinic. In view of this fact I have described below the results of the tests where different types of fruits, which ripen in different months, were tested, depending on the season in which the tests were made, divided into two series, the colder and the warmer season.

III. Test results

I. Formation of glycogen from infused glucose in the colder seasons.

In order to obtain the control value for the amount of glycogen

formed in the liver from infused glucose, a test was made in which the rabbits received intravenously, as mentioned above, per kg 10 cc 25 g/dl sugar solution 12 hours after the provisional glycogen-eliminating lecithin infustion, and were killed after an additional three hours. The value of 1.16% found represents the amount of glycogen which was formed in the liver from infused glucose.

Table 1. Glycogen Content of the Liver of Healthy Rabbits After Supply of Glucose.

3 Hours After Intravenous Infusion of 2.5 g Glucose.(Oct. 2-3, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
1	1940	1910	55	0.92
2	1750	1690	47	1.08
3	1710	1620	39	1.38
4	1810	1750	49	1.22
5	1620	1510	58	1.21
average				1.16

2. Enhancing effect of l-ascorbic acid on the formation of glycogen

In a test as described above we injected subcutaneously together with the sugar infusion per kg 1 cc redoxon which contained 50 mg l-ascorbic acid as sodium salt. The glycogen yield was 1.90%, it thus increased by the addition of ascorbic acid by 63%, which verified to a certain extent the observation by Watanabe (5).

Table 2. Glycogen Content of the Liver After Supply of l-ascorbic Acid and Glucose.

3 Hours After the Supply of 50 mg l-Ascorbic Acid and 2.5 g Glucose (Oct. 5-6, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
6	2080	1940	49	1.69
7	1540	1360	44	1.92
8	1890	1700	55	1.80
9	2100	1930	47	2.24

(Table con't)

10	2400	2060	63	1.86
Average				1.90

3. Enhancing effect of d-araboascorbic acid on the formation of glycogen.

In this test we used with an otherwise identical arrangement instead of redoxon the same amount of viton which contains 50 mg d-araboascorbic acid per cc.

Table 3. Glycogen Content of the Liver After Supply of d-Araboascorbic Acid and Glucose.

3 Hours After the Supply of 50 mg d-Araboascorbic acid and 2.5 Glucose (Oct. 15-16, 1937)

Rabbit no.	body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
11	1930	1770	65	1.89
12	1520	1470	55	1.61
13	1620	1550	57	2.18
14	1770	1650	52	1.79
15	1760	1620	55	1.79
Average				1.87

The value for liver glycogen found was 1.87%, which was 61% higher than the control value. d-araboascorbic acid has thus almost the same enhancing effect on the formation of glycogen as l-ascorbic acid, though it is much weaker than the latter in its anti-scurvy effect, as known.

4. Effect of a mixture of l-ascorbic acid and glucose on the formation of glycogen

In order to test whether l-ascorbic acid is stable in glucose solution, we dissolved 50 mg of the substance in 10 cc of a 25 g/dl glucose solution, sterilized it under exclusion of air, and stored it. The solution was injected intravenously into the rabbits in an amount of 10 cc per kg, causing the formation of 1.68% liver-glycogen. This value is perhaps lower than in the test where the individual substances were infused separately (see test 2), but it is higher by 45% than the control value.

Table 4. Glycogen Content of the Liver After Infusion of a Mixture of l-Ascorbic Acid and Glucose.

3 Hours After the Infusion of 50 mg l-Ascorbic Acid and 2.5 g Glucose. (Oct. 21-22, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
16	1770	1640	58	1.77
17	1940	1890	61	1.45
18	1560	1550	48	1.85
19	1730	1530	59	1.86
20	1630	1510	47	1.47
Average				1.68

5. The same test as above with d-araboascorbic acid.

In the same test as above we used instead of l-ascorbic acid 50 mg d-araboascorbic acid with a glucose yield of 1.66%, which was likewise somewhat lower than in the corresponding test with separate infusion, but which was 43% higher than the control value. Since l-ascorbic acid and d-araboscorbic acid proved equivalent in these last two tests in their effect on the glycogen yield, we used in the following tests exclusively d-araboascorbic acid instead of l-ascorbic acid, because we had to exclude at that time expensive patented substances, in view of the war shortages.

Table 5. Glycogen Content of the Liver After Infusion of a Mixture of d-Araboascorbic Acid and Glucose.

3 Hours After the Infusion of 50 mg d-Araboascorbic Acid and 2.5 g Glucose (Oct. 24-25, 1937).

Rabbit no.	Weight of body (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
21	1660	1550	41	1.80
22	1760	1630	65	1.48
23	1620	1410	45	1.89
24	1880	1580	55	1.45
25	2180	2030	56	1.69
Average				1.66

6. Effect of lemon juice on the formation of glycogen (citrus medica

L. Subsp. Limmonum Hook).

For the tests, a mixture of 10 cc 25 g/dl glucose solution and 1 cc fresh lemon juice per kg were injected into the rabbits intervenously, after which a liver glycogen content of 2.17% was found (table 6). This means that the glucose yield resulting from injected sugar had increased by 90% by the addition of lemon juice and the enhancing effect of only 1 cc lemon juice on the formation of glycogen is greater than that of 50 mg d-ascorbic acid.

Table 6. Glycogen Content of the Liver After Infusion of Glucose With Fresh Lemon Juice.

3 Hours After the Infusion of Lemon Juice 1 cc and Glucose 2.5 g. (Nov. 1-2, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
26	1930	1750	52	1.74
27	1920	1690	56	2.52
28	1940	1850	58	2.24
29	1830	1740	47	2.28
30	2150	1930	58	2.06
Average				2.17

In the second test we added again per kg 1 cc lemon juice to the same mixture of glucose and d-araboascorbic acid as it was used in test no. 5. The glycogen yield thus increased to 3.06% (table 7), compared to the test without the addition of lemon juice it thus increased by 90% (see test no. 5). The vitamin C content of the lemon juice used was in reducing form 47.6 mg% and in the total amount 48.8 mg%, hence 1 cc juice, which was available in this test, contains 0.5 mg vitamin, which, though it is equivalent as a whole to ascorbic acid, represents only one hundredth of the amount of the same substance used in the foregoing tests (see test no. 2 and 4). From this viewpoint we have to assume the presence of another still unknown substance, apart from ascorbic acid, for the explanation of the unexcelled effect of fresh lemon juice in the formation of glycogen.

One can naturally doubt if the reducing substances, particularly the sugars which are contained in lemon juice and which can be available as a source of the formation of new glycogen, have contributed to the increase of the glycogen yield. Such an increase of the glycogen yield, which actually existed, can be readily ignored, however, if we consider that the amount of the reducing substances in 1 cc lemon juice, which resulted from my determination corresponding to only 0.05 g glucose, is out of the question when working with an infusion of 2.5 g sugar per kg.

In the third test we investigated the property of the above mentioned new substance whether it can pass over it into alcohol in the active state. To this end we mixed 1 volume of fresh lemon juice with 5 volumes 95% alcohol, stored the mixture cold and filtered after 2 weeks. The filtrate was concentrated under reduced pressure at 60°C to 1/3 volume. The extract thus produced corresponds a double dilution of the fresh lemon juice. 2 cc of this extract were injected intravenously into the rabbits together with 10 cc of the same mixture of glucose and d-araboascorbic acid which was used in test no. 5, which resulted in a glycose yield of 2.47% (table 8). This value is lower than in the test with fresh lemon juice, but by 50% higher compared to the test without the addition of lemon juice. This means that the substance of the lemon juice in question, which enhances the formation of glycogen, passes over into alcohol and is rather stable.

Table 7. Glycogen Content of the Liver After Infusion of "d-Araboascorbic Acid + Glucose" After the Infusion, of 1 cc Lemon Juice, d-Araboascorbic Acid mg, Glucose 2.5 g. (Nov. 2-3, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
31	1860	1730	59	3.98
32	1740	1590	53	4.03
33	1520	1480	48	2.44
34	1790	1540	50	1.88
35	1630	1420	48	2.95
Average				3.06

Table 8. Glycogen Content of the Liver After Infusion of "d-Araboascrobic Acid + Glucose" With Alcohol Extract of Lemon Juice.

2 Hours After Infusion. Alcohol Extract of Lemon Juice 2 cc, d-Araboascorbic Acid 50 mg, Glucose 2.5 g. (Nov. 15-16, 1937).

Rabbit no	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
36	1880	1730	50	2.12
37	1920	1890	60	1.90
38	1610	1470	63	1.66
39	2220	1910	74	3.92
40	2210	1900	69	2.77
Average				2.47

7. Test with juice of tangerine (Citrus aurantium L subsp. nobilis Makino).

When fresh tangerine juice and glucose were used under otherwise equal conditions, as in the preceding tests, liver glycogen was found in an amount of 2.02% (table 9), but in another test it increased to 2.43% by the addition of d-araboascorbic acid (table 10). The formation of glycogen from [illegible]sed sugar thus increases by 45% and 80% resp. by the addition of fresh tangerine juice.

Table 9. Glycogen Content of the Liver After Infusion of Glucose and Fresh Tangerine Pulp Juice.

3 Hours After Infusion. Tangerine Pulp Juice 1 cc, Glucose 2.5 g. (Nov. 7-8, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
41	1590	1450	55	1.87
42	1900	1750	62	2.27
43	1730	1560	55	1.77
44	1710	1540	50	2.31
45	1650	1530	52	1.90
Average				2.02

Table 10. Glycogen Content of the Liver After Infusion of d-Araboascorbic Acid + Glucose and Fresh Tangerine Juice.

9 Hours After Infusion. Tangerine Juice 1 cc, d-Araboascorbic Acid 50 mg,

Glucose 2.5 g. (Nov. 8-9, 1937).

Rabbit no.	Body Weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
46	1680	1570	67	2.44
47	1700	1600	45	2.61
48	1530	1480	55	2.08
49	1600	1460	52	2.28
50	1610	1540	56	2.76
Average				2.43

By using an alcohol extract instead of fresh tangerine juice, the liver glycogen of the rabbits which had been treated at the same time with d-araboascorbic acid and glucose dropped to 2.24% (table 11), which was still by 35% higher than in the tests without the addition of the extract (see test no. 4). The vitamin C content of the tangerine juice was 29.4 mg% in reducing form and 33.6% in the total amount.

Table 11. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with alcohol extract of tangerine juice.

3 Hours after infusion. Alcohol extract of tangerine juice 2 cc, d-araboascorbic acid 50 mg, glucose 2.5 g.

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver	Liver glycogen (%)
51	1700	1660	57	1.91
52	1760	1680	62	2.99
53	1720	1690	57	2.21
54	1630	1440	59	1.68
55	1500	1430	45	2.41
Average				2.24

8. Test with juice of bitter orange (Citrus aurantium L. subsp. Emara Engl.)

When the fresh juice of bitter orange was supplied at the same time, the amount of glycogen formed from infused sugar in the liver was 1.60%, hence 27% higher than the control value (table 12). When d-araboascorbic acid was used, the liver glycogen content was 2.12%, due to the cooperation of the fresh juice (table 13), and 1.99% by the addition of alcohol extract instead of fresh juice (table 14). The juice of the bitter orange was thus

less effective in enhancing the formation of glycogen than lemon- and tangerine juice. The vitamin C content of the juice used could be determined in reducing form with 24.4 mg% of a total amount of 25.5 mg%.

Table 12. Glycogen content of the liver after infusion of glucose with fresh pulp juice of the bitter orange.

3 hours after infusion. Pulp juice of the bitter orange 1 cc, glucose 2.5 g. (Dec. 1-2, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
56	1680	1580	55	1.62
57	1450	1370	44	1.54
58	1660	1520	52	1.48
59	1710	1540	50	1.64
60	1710	1570	55	1.72
Average				1.60

Table 13. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with fresh juice of the bitter orange.

3 hours after infusion. Pulp juice of the bitter orange 1 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (Dec. 2-3, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
61	1870	1780	60	1.88
62	1680	1550	67	2.02
63	1590	1520	47	2.54
64	1730	1630	54	2.17
65	1740	1540	55	1.97
Average				2.12

Table 14. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with alcohol extract of the juice of the bitter orange.

3 hours after infusion. Alcohol extract of the juice of the bitter orange 2 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (dec. 5-6, 1937)

Rabbit no.	Weight body (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
66	1900	1770	69	2.28
67	1810	1700	58	1.97

(Table con't)

68	1840	1650	59	1.82
69	1660	1520	55	2.09
70	1710	1640	52	1.77
Average				1.99

9. Test with grapefruit (Citruc Sabon Sieb).

1.73% liver glycogen was obtained by the infusion of fresh grapefruit juice and glucose, which was by 50% higher than the control value (table 15). By adding d-araboascorbic acid to the infusion, the glycogen yield increased to 1.97% (table 16). When alcohol extract was used instead of fresh juice, it dropped again to 1.81% (table 17). In the second test series the pressed juice of the grape fruit peel was used instead of the pulp juice. By adding 1 cc of the same juice per kg to the glucose solution to be infused, we obtained a liver glycogen content of 1.79% (table 18), which was almost as high as in the test with pulp juice. The simultaneous infusion of d-araboascorbic acid increased the glycogen yield to 2.05% (table 19). The replacement of the fresh juice by alcohol extract under otherwise identical conditions reduced the glycogen liver content to 1.85% (table 20). The vitamin C content of the pulp juice was 35.7 mg% in reducing form and 37.5 mg% in the total amount, that of the peel juice was 66.7 mg% and 67.2 mg% resp. As known, grapefruit peel contains much more

Table 15. Glycogen content of the liver after infusion of glucose with fresh grapefruit pulp juice.

3 hours after infusion. Grapefruit pulp juice 1 cc, glucose 2.5 g (Dec. 8-9, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
71	1750	1610	62	1.68
72	1730	1640	62	1.50
73	1860	1780	64	1.89
74	1660	1580	52	1.84
75	1630	1520	48	1.76
Average				1.73

Table 16. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with fresh grapefruit pulp juice.

3 hours after infusion. Grapefruit pulp juice 1 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (Dec. 9-10, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
76	1760	1710	75	2.12
77	1720	1630	60	2.24
78	1750	1720	72	1.82
79	1970	1860	67	1.81
80	1830	1790	60	1.84
Average				1.97

Table 17. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with alcohol extract of grapefruit pulp juice.

3 hours after infusion. Alcohol extract of grapefruit pulp juice 2 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (Dec. 22-23, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
81	1540	1440	45	1.77
82	1510	1500	45	2.00
83	1570	1470	52	2.06
84	1590	1460	50	1.54
85	1720	1620	54	1.66
Average				1.81

Table 18. Glycogen content of the liver after infusion of glucose with fresh grapefruit peel juice.

3 hours after infusion. Grapefruit peel juice 1 cc, glucose 2.5 g. (Dec. 25-26, 1937).

Rabbit no	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
86	1650	1630	60	2.00
87	1740	1690	47	1.58
88.	1520	1480	57	1.70
89	1690	1640	87	1.92
90	1420	1370	42	1.75
Average				1.79

Table 19. Glycogen content of the liver after infusion of "d-araboascorbic

acid + glucose" with grapefruit peel juice.

3 hours after infusion. Grapefruit peel juice 1 cc, d-araboascorbic acid 50 mg, glucose 2.5 g, .(Dec. 26-27, 1937).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
91	1850	1650	60	1.78
92	1580	1520	44	2.12
93	1700	1580	65	2.00
94	1540	1400	49	1.97
95	1760	1620	59	2.40
Average				2.05

Table 20. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with alcohol extract of grapefruit peel juice.

3 hours after infusion. Alcohol extract of grapefruit peel juice 2 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (Jan. 9-10, 1938).

Rabbit no	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
96	1820	1570	62	2.01
97	1720	1620	57	1.86
98	1670	1550	55	2.13
99	1480	1470	45	1.75
100	1540	1430	50	1.50
Average				1.85

vitamin than the pulp and in fact the former proved to be about twice as high as the latter according to my determination, nevertheless the two components are about equal as far as their enhancing effect on the formation of glycogen is concerned, which is due to the fact that the glycogen-enriching effect in question does not always go parallel with the vitamin C content.

10. Test with apple juice

Fresh apple juice, added to the glucose solution and infused, caused a glycogen yield in the liver of rabbits of 1.73% (table 21). Addition of d-araboascorbic acid to the infusion increased the yield to 2.38% (table 22), which dropped, however, to 2.17% if the fresh juice was replaced by

alcohol extract (table 23).

The apple proved little effective against scurvy according to investigations by Holst and Froelich (21). In agreement with this finding are the observations by Tillmans (22) and Fujita (23) that the vitamin C content of the apple is very low in general. In my investigation I found practically no reducing form, but a total amount of 5.3 mg%, which corresponds to about 1/9 of the lemon juice and 1/5 of the bitter orange juice. Nevertheless apple juice exceeds the bitter oranges in their glycogen-enriching effect, though it is slightly below that of the lemons. We can also see at this point that the effect of the fruits on the glycogen content gows by no means parallel with their vitamin content.

Table 21. Gylcogen content of the liver after infusion of glucose with fresh apple juice.

3 hours after infusion. Apple juice 1 cc, glucose 2.5 g. (Jan. 15-16, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
101	2360	2180	70	1.96
102	1710	1540	57	1.72
103	1630	1450	47	1.78
104	1390	1330	40	1.63
105	1700	1640	58	1.56
Average				1.73

Table 22. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with fresh apple juice.

3 hours after infusion. Apple juice 1 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (Jan. 15-16, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
106	1530	1360	45	2.51
107	1620	1520	50	2.42
108	1640	1600	49	2.66
109	1810	1690	72	2.08

(Table con't)

110	1730	1570	47	2.25
Average				2.38

Table 23. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with alcohol of apple juice.

3 hours after infusion. Alcohol extract of apple juice 2 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (Jan. 30-31, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
111	1420	1380	39	2.35
112	1630	1570	67	1.85
113	1530	1430	51	2.56
114	1450	1270	43	1.98
115	1690	1620	62	2.09
Average				2.17

11. Test with green tea infusion

5 g green tea (Thea sinensis L.) were digested with 100 cc water at 50°C for 30 minutes and filtered. 1 cc of the filtrate per kg was used for the infusion with the usual amount of glucose. The glycogen content was 1.71% (table 24), when d-araboascorbic acid was added it increased to 1.89% (table 25).

That green tea is very rich in vitamin C was already verified by Miura and Tuzimura (24). Miura (25) also pointed out that a too hot extraction results in a loss of vitamin. Since I found in my tests for the reducing vitamin form 250 mg% and a total amount of 278.6 mg% in the green tea substance, and 13.5 mg% and 15.6 mg% resp. in a 20x diluted infusion, it is clear that the entire vitamin amount was extracted. As far as the glycogen enrichment is concerned, the gree tea infusion is relatively little effective, in contrast to its rich vitamin content.

Table 24. Glycogen content of the liver after infusion of glucose with green tea infusion.

3 hours after infusion. Green tea infusion 1 cc, glucose 2.5 g. (Feb.

4-5, 1938)

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
116	1990	1780	44	1.72
117	2050	1770	60	2.01
118	1550	1460	48	1.86
119	1630	1520	55	1.37
120	2040	1960	59	1.57
Average				1.71

Table 25. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with green tea infusion.

3 hours after infusion. Green-tea infusion 1 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (Feb. 5-6, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
121	1640	1530	55	1.74
122	1850	1710	52	1.85
123	1890	1760	54	2.19
124	1840	1680	42	1.78
125	1950	1850	59	1.88
Average				1.89

12. Effects of flavone compounds on the formation of glycogen.

For the tests were used flavone compounds, like l-matteucinol, homoeryodictyol, desmethoxymatteucinol, and 7-oxyflavanone, which belong to the flavanone group. l-matteucinol in an amount of 2 mg per kg was dissolved in 1 cc 95% alcohol and mixed with 10 cc 25 g/dl glucose and injected intravenously into the rabbits so that a glucose yield of 1.49% was obtained (table 26). This value is only by 28% higher than the control value with sugar infusion alone. By adding d-araboascorbic acid, the yield was increased to 2.47% (table 27), which is by 48% higher than the value found without l-matteucinol.

In a similar test arrangement we obtained a glycogen yield of 1.35% when using homoeryodctyol and glucose (table 28), and of 1.82% when d-araboascorbic acid was added (table 29).

Homoeryodictyol thus did not markedly enhance the formation of glycogen. Somewhat more effective, however, were two other flavone compounds. Desmethoxymatteucinol and glucose caused 1.55% glycogen (table 30), by adding d-araboascorbic acid the glycogen content increased even to 2.03% (table 31). 7-oxyflavanone and glucose brought a glycogen yield of 1.54% (table 32), which increased to 2.25% (table 33) when d-araboascorbic acid was added. Of the 4 flavone compounds used in the test, l-matteucinol and 7-oxyflavanone rank first as far the glycogen-enriching effect is concerned, followed by desmethoxymatteucinol and last by homoeryodictyol.

Table 26. Glycogen content of the liver after infusion of glucose with l0matteucinol.

3 hours after infusion, l-matteucinol 2 mg, glucose 2.5 g. (Feb. 12-13, 1973).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
126	1640	1520	45	1.33
127	1890	1820	61	1.28
128	1650	1630	66	1.78
129	1570	1520	49	1.39
130	1630	1440	59	1.68
Average				1.49

Table 27. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with l-matteucinol.

3 hours after infusion. l-matteucinol 2 mg, d-araboascorbic acid 50 mg, glucose 2.5 g. (Feb. 22-23, 1938).

Rabbit no.	Body weight (g) in the beginning	in the end	Weight of liver (g)	Liver glycogen (%)
131	1680	1510	52	1.91
132	1620	1470	47	2.80
133	2130	1850	74	2.68
134	1640	1470	65	2.02
135	1440	1320	47	2.93
Average				2.47

Table 28. Glycogen content of the liver after infusion of glucose with homoeryodictyol.

3 hours after infusion. Homoeryodictyol 2 mg, glucose 2.5 g. (March 1-2, 1938).

Rabbit no	Body weight (g) at the beginning	at the end	Weight of Liver(g)	Liver glycogen (%)
136	1650	1560	52	1.44
137	1680	1500	55	1.26
138	1750	1620	40	1.17
139	1510	1400	40	1.17
140	1750	1700	54	1.22
Average				1.35

Table 29. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with homoeryodictyol.

3 hours after infusion. Homoerydictyol 2 mg, d-araboascorbic acid 50 mg, glucose 2.5 g. (March 4-5, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
141	1840	1660	57	1.58
142	1890	1600	56	2.10
143	1530	1420	43	1.66
144	1630	1530	61	1.86
145	1620	1450	60	1.92
Average				1.82

Table 30. Glycogen content of the liver after infusion of glucose with desmethoxymatteucinol.

3 hours after infusion. Desmethoxymatteucinol 2 mg, glucose 2.5 g. (March 8-9, 1939)

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
146	1660	1580	57	1.39
147	1860	1760	67	1.20
148	1830	1700	57	1.73
149	1430	1370	50	1.90
150	1370	1850	42	1.54
Average				1.55

Table 31. Glycogen content of the liver after infusion of "d-araboascorbic

acid + glucose" with desmethoxymatteucinol.

3 hours after infusion. Desmethoxymatteucinol 2 mg, d-araboascorbic acid 50 mg, glucose 2.5 g. (March 11-12, 1938)

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
151	1850	1700	58	1.50
152	1800	1510	54	2.05
153	1630	1520	45	1.33
154	1410	1320	41	2.41
155	1950	1790	62	2.93
Average				2.03

Table 32. Glycogen content of the liver after infusion of glucose with 7-oxyflavonone.

3 hours after infusion. 7-oxyflavanone 2 mg, glucose 2.5 g. (March 14-15, 1938)

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
156	1660	1650	50	1.36
157	1500	1400	47	1.45
158	1710	1670	65	1.61
159	1430	1420	40	1.80
160	1610	1570	57	1.50
Average				1.54

Table 33. Glycogen content of the liver after infusion of "d-arabascorbic acid + glucose" with 7-oxyflavanone.

3 hours after infusion. 7-oxyflavanone 2 mg, d-araboascorbic acid 50 mg, glucose 2.5 g. (March 20-21, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
161	1510	1400	48	2.92
162	1910	1840	56	1.90
163	1610	1380	55	2.91
164	1700	1530	56	1.84
165	1620	1400	47	1.68
Average				2.25

13. Control values of the glycogen content of the rabbits for warmer seasons.

Since the fruits which ripen in the warmer seasons were available for the following tests, the control values of the glycogen content of the animals had to be investigated again for these seasons.

A test made in June shows that, after the infusion of the usual amount of glucose, a glycogen content of 0.98% was found on the average in the liver. This value is only by 17% lower than in the above described October test.

In a second test, likewise made in June, where glucose was used together with d-araboascorbic acid, we found a glycogen yield of 1.47%, which is 13% less than in the October test. By adding d-araboascorbic acid, the yield could be increased, however, by 50%.

Table 34. Glycogen content of the liver after infusion of glucose.

2 hours after infusion of 2.5 g glucose.

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
166	1610	1520	48	1.12
167	1590	1540	45	0.95
168	1790	1620	57	0.88
Average				0.98

Table 35. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose."

3 hours after infusion. d-araboascorbic acid 50 mg, glucose 2.5 g. (June 6-7, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
169	1650	1560	60	1.44
170	1830	1730	48	1.38
171	1730	1550	44	1.58
Average				1.47

14. Tests with summer orange juice (citrus aurantium l. subsp. Natsudaidai, Hayata).

When glucose was used with 1 cc summer orange juice, 1.25% liver

glycogen was found (table 36); when d-araboascorbic acid was added the value increased to 1.83% (table 37), which was further increased, however, when the pulp juice was replaced by pressed juice from the peel. The peel juice is thus more effective, as far as the deposit of glycogen is concerned, than the pulp juice. The former contained 55.6 mg% of the reducing form in the total vitamin C amount of 59.1 mg%, while in the latter the values were 20.8 mg% and 23.8 mg% resp. The effect of the two constituents on the glycogen content thus seems to correspond to the vitamin content. But this must probably be considered an accidental finding.

Table 36. Glycogen content of the liver after infusion of glucose with fresh pulp juice of the summer orange.

3 hours after infusion. Pulp juice of the summer orange 1 cc, glucose 2.5 g. (June 10-11, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
172	1510	1350	47	1.24
173	1690	1580	50	1.18
174	1620	1510	49	1.32
Average				1.25

Table 37. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with pulp juice of the summer orange.

3 hours after infusion. Pulp juice of summer orange 1 cc, d-araboascorbic acid, 50 mg, glucose 2.5 g. (June 10-11, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
175	1630	1520	58	1.74
176	1690	1430	53	2.08
177	1460	1360	40	1.68
Average				1.83

Table 38. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with fresh juice of the summer orange peel.

3 hours after infusion. Juice of the summer orange peel 1 cc, d-arabo-

ascorbic acid 50 mg, glucose 2.5 g. (June 10-11, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
178	1600	1530	44	1.89
179	1730	1640	57	2.20
180	1650	1520	48	3.00
Average				2.36

15. Test with strawberry juice

The usual amount of glucose with 1 cc fresh strawberry juice caused a glycogen yield of 1.33% (table 39), the addition of d-araboascorbic acid increased the latter to 2.09% (table 40).

Strawberry juice is very rich in vitamin C, as known, and the determination yielded the reducing form of 62 mg% in a total amount of 75 mg%.

Table 39. Glycogen content of the liver after infusion of glucose with fresh strawberry juice.

3 hours after infusion. Fresh strawberry juice 1 cc, glucose 2.5 g. (June 20-21, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
181	1820	1730	47	1.34
182	1740	1630	52	1.38
183	1500	1320	40	1.27
Average				1.33

Table 40. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with fresh strawberry juice.

3 hours after infusion. Fresh strawberry juice 1 cc, d-araboascorbic acid 50 mg, glucose 2.5 g. (June 20-21, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
184	2210	2070	57	1.96
185	2070	2060	55	2.31
186	1980	1840	50	2.01
Average				2.09

16. Test with tomato juice

Glucose with 1 cc fresh tomato juice and injected yielded a glycogen content of 1.46% in the liver (table 41), which the addition of d-arabo-ascorbic acid increased this value to 2.26% (table 42). By using extract instead of fresh fruit juice, the glycogen yield dropped to 1.71% (table 43). The determination of the vitamin content yielded 17.9 mg% in reducing form of a total amount of 21 mg%. These values are less than one third of the vitamin content of strawberry juice, despite the greater effect on the glycogen content.

Table 41. Glycogen content of the liver after infusion of glucose with fresh tomato juice.

3 hours after infusion. Fresh tomato juice 1 cc, glucose 2.5 g. (July 13-14, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
187	1590	1700	44	1.52
188	1560	1410	41	1.45
189	1560	1440	40	1.40
Average				1.46

Table 42. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with tomato juice.

3 hours after infusion. Tomato juice 1 cc, d-araboascorbic acid 5 mg, glucose 2.5 g. (July 13-14, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
190	1810	1700	53	2.18
191	1770	1670	54	2.43
192	2110	1960	60	2.16
Average				2.26

Table 43. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with alcohol extract of tomato juice.

3 hours after infusion. Alcohol extract of tomato juice 2 cc, d-arabo-ascorbic acid 50 mg, glucose 2.5 g. (July 29-30, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
193	1650	1570	47	1.79
194	1630	1480	50	1.72
195	1580	1510	49	1.62
Average				1.71

17. Test with water melon juice (citrullus vulgaris Schrad).

A mixture of glucose and 1 cc fresh watermelon juice yielded a liver glycogen content of 1.52% (table 44) by adding d-araboascorbic acid the yield increased to 2.29% (table 45). The amount of vitamin C in the water-melon juice was 3.6% in reducing form of a total amount of 4.9 mg%. The relatively low vitamin content in the melon juice exceeds, however, in its glycogen enrichment strawberry juice whose vitamin content is about 15 times higher.

Table 44. Glycogen content of the liver after infusion of glucose with fresh watermelon juice.

3 hours after infusion. Fresh watermelon juice 1 cc, glucose 2.5 g. (Aug. 3-4, 1939).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
196	1520	1490	52	1.26
197	1650	1600	47	1.56
198	1590	1500	42	1.74
Average				1.52

Table 45. Glycogen content of the liver after infusion of "d-araboascorbic acid + glucose" with fresh watermelon juice.

3 hours after infusion. Fresh watermelon juice 1 cc, d-araboascorbic acid 50 mg, glucose, 2.5 g. (Aug. 3-4, 1938).

Rabbit no.	Body weight (g) at the beginning	at the end	Weight of liver (g)	Liver glycogen (%)
199	1810	1670	50	1.63
200	1610	1500	60	2.70
201	1680	1580	49	2.55
Average				2.29

IV. Discussion

In preceding tests we verified the finding, noticed first by Alternburger (3) and Watanabe (5), that l-ascorbic acid enhances the formation of glycogen from infused sugar even in animals not suffering from scurvy. The carriers of this glycogen-enriching effect, however, are not limited according to my investigation to l-ascorbic acid alone, they are also found among the related chemical substances, and even among the natural products of the vegetable kingdom.

Among the former substances is particularly d-araboascorbic acid, which is said to be much less effective against scurvy than l-ascorbic acid, according to investigations by preceding authors. This isomer of l-ascorbic acid proved to be just as effective for the glycogen yield of the liver as l-ascorbic acid. For this reason alone it can be assumed that the glycogen-enrichening effect of these substances is not identical with the anti-scurvy effect. If we limit the definition of vitamin C to an anti-scurvy substance, the effect relating to the glycogen yield will naturally not be included in the respective vitamin. I therefore tentatively call the phenomenon in question in this paper the vitamin C_3 effect, since the name C_2 was already given by Euler (26) to the antipneumonic factor which is said to occur in lemon juice without prejudice to the present of substances with a different composition than vitamin C proper or better vitamin C_1, because these substances, like l-ascorbic acid or d-araboascorbic acid have both an anti-scurvy effect and a glycogen-enriching effect, as it results from the above described tests.

The incongruence of the anit-scurvy effect and of the glycogen-enriching effect, that is, of the vitamin C_1 and vitamin C_3 effect, can be observed already from these synthetically produced substances. In this paper we have also investigated the vitamin C_3 effect with different vegetable substances which could always be demonstrated more or less clearly.

But their strength was frequently not in agreement with the vitamin C contents, which were determined with the dichlorophenol-indophenol-method introduced by Tillmanns (18). An example will perhaps clarify this situationcompletely. As mentioned above, a higher glycogen yield can be achieved in a sugar infusion by adding 1 cc fresh lemon juice than by adding 50 mg l-ascrobic acid, which represents an optimum amount according to investigations by Watanabe (5). But since 1 cc lemon juice contains only about 0.5 mg vitamin C, there is no doubt that the enhancing effect of lemon juice on the formation of glycogen depends not on its vitamin C content alone, we must rather assume that a specific factor exists in the natural vegetable products which is certainly different from the ascorbic acid known to us, and whose enriching effect on the formation of glycogen is over 100 times greater than the latter.

The effects of the vegetable substances on the glycogen yield are rather complicated, however, which will be discussed later. Here we only want to consider the effects of the simple chemical substances. As known, Szent-Gyoergyi (10) isolated recently from lemon juice a sort of flavone glycoside which the author suggested to call vitamin P, because of its effect on the permeability of the vessels. Whether this new substance has any effect on the glycogen yield, we do not know yet. At any rate the flavone glycosides are widespread in the vegetable kingdom.

For my investigations I used the flavone compounds l-matteucinol, homoeryodictyol, desmethoxymatteucinol and 7-exyflavanone. By using glucose + l-matteucinol we obtained 0.33% more glycogen than from sugar alone, so that this amount must be due directly the effect of l-matteucinol. By using clucose- + d-araboascorbic acid + l-matteucinol we obtained 2.47% glycogen (see test no. 12), which is by 0.48% higher than the calculated sum of 1.66%, which resulted from test no. 4 with glucose + d-araboascorbic acid, plus 0.33%, which represents, as mentioned above, the yield of the

direct effect of l-matteucinol in the sugar infusion. This additional amount of 0.48% can perhaps be considered a result of the interrelations existing between l-matteucinol and d-araboascorbic acid, so that their actions are increased unilaterally or mutually. Such an additional amount of glycogen, which exceeds the calculated sum, was found also when we used glucose + d-araboascorbic acid + 7-oxyflavanone, though it was not more than 0.21%. In the test with homoeryodictyol or desmethoxymatteucinol we can also demonstrate more or less clearly a directly enhancement of the glycogen yield, but no mutual increase of the effects between these substances and d-araboascorbic acid, because the glycogen yields found when these substances were used simultaneously were always in good agreement with the calculated sum of the values taken from the individual samples. We thus arrive at the conclusion that the four types of flavone compounds which are used for the investigation all enhance directly the formation of glycogen from infused sugar, but as far as the properties of increasing the effect of d-araboascorbic acid on the formation of glycogen are concerned they can only be found in two of them.

In the effects of the chemical flavone compounds on the glycogen yield we can recognize two factors, namely one which enhances the formation of glycogen directly (factor I) and a second one which increases the respective effect of the other substances (factor II). These effects can naturally also be found in the natural products of the vegetable kingdom in which the glycosides occur, which contain the flavone compounds as agrykon. In order to illustrate this question I will consider more closely the individual results of the tests with vegetable products.

When using 1 cc lemon juice with sugar solution we found 2.17% liver-glycogen (see table 6), which is by 1,01% higher than the value in the test, without lemon juice. This additional amount is thus due to the effect of factor I. In the test with lemon juice + d-araboascorbic acid we found a glycogen yield of 3.06%, which is 0.39% higher than the calculated sum of

1.66% from sugar + d-araboascorbic acid and 1.01% from the direct effect of the lemon juice. This difference between the amount of glycogen found and calculated must be considered a result of the ascorbic acid effect increased by factor II. This way there are 2 factors of vitamin C in lemon juice.

With this type of calculation we obtain with strawberries as the effect of factor I 0.35%, and of factor II 0.27, with tomatoes 0.48% and 0.31% resp. and with watermelon 0.54% and 0.28%, all of which thus belong to the category which carries both factors of vitamin C_3.

Slightly different is the behavior of the other group of vegetable products investigated by us. Here the presence of factor I could always be demonstrated, while factor II was missing or was not observed clearly. For example, in apple juice we found 0.57% glycogen as an effect of factor I. The glycogen yield found in the test with apple juice + d-araboascorbic acid + sugar was 2.38%, which was only slightly higher than the calculated sum of 2.23%.

In the test with apple juice, we can calculate as the effect of factor II a small but still positive value. In the other vegetable substances, however, we always obtain a more or less negative value, namely with tangerine juice a glycogen yield of 0.86% as the effect of factor I, in contrast to -0.09% for factor II. In bitter orange, grapefruit, summer orange and green tea, we observed likewise only negative values for factor II, though factor I caused a more or less abundent glycogen yield.

The various natural products of the vegetable kingdom, just like the chemical flavone compounds, thus cause only partly a direct enrichment of the deposited glycogen, while they partly increase in addition the glycogen-enriching effect of the other substances. Only by assuming these complicated processes can we explain the observation that an amount of fresh lemon juice exceeds in its glycogen-enriching effect a 100 times larger amount of

l-ascorbic acid, as far as the vitamin C content is concerned, which latter represents the pure vitamin C substance.

The results of the vitamin C determination are generally in good agreement with those of the preceding authors. The different types of oranges are rich in vitamin C, and particularly their peels are richer than the pulp. As far as oranges are concerned, the glycogen yield was found increased corresponding to their vitamin C content, but this does not mean that the glycogen effect is completely independent of the vitamin content, because these two part company in tests with other fruits. Apple and watermelon, which are very low in vitamin C, exceed by far the vitamin-rich strawberry juice in their glycogen-enriching effect. This observation, together with the other results described above, which likewise indicate the incongruence of the vitamin content with the glycogen effect, leads us to the assumption of the existence of other factors in the vegetable substances which cause the glycogen enrichment, in addition to l-ascorbic acid. Details of these factors, particularly if they are related to the vitamin P described by Szent-Gyoergyi (10), need further clarification.

V. Conclusion

1. l-ascorbic acid enhances the formation of glycogen in the liver, which effect is also shown in the same intensity by its isomer d-arabo-ascorbic acid.

2. The same direct glycogen effect is also found in the various vegetable products, like fruits and green tea, but their strength does not always go parallel with the vitamin C content. A close relationship between the glycogen-enriching effect and the vitamin C content is not very likely because a small amount of vegetable substances, which correspond in terms of the vitamin C content to several tenths or a hundredth of the optimum amount of l-ascorbic acid, cause usually such an enormous glycogen yield that it far exceeds the amount that can be released by the latter.

3. The vegetable products carry in addition other factors, which greatly enhance the glycogen-enriching effect of other substances, like l-ascorbic acid or d-araboascorbic acid, so that the glycogen yield is further increased. But not every vegetabel substances is provided with this indirect effect, in contrast to the direct effect, which is widespread among the natural vegetable products.

Similar effects as the vegetable products have on the glycogen yield, particularly a direct and an indirect effect, are also observed in the chemically prepared flavone compounds, but the indirect effect appears only in some of them.

References

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Isoascorbic Acid, Sodium Sal
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Wirkungen des Vitamins C und der verwandten Stoffe auf den Glykogenbestand.

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I. Einleitung.

Dass das Vitamin C, der bekannte antiskorbutische Stoff, auch auf den Kohlehydratstoffwechsel eine Wirkung ausübt, ist schon von manchen Autoren angegeben worden. Stepp, Schroeder und Altenburger[1] und Stoïcesco und Gingold[2] fanden so bei Gesunden eine Senkung des Blutzuckers nach intravenöser Applikation der l-Ascorbinsäure. Altenburger[3] beobachtete bei skorbutischen und auch normalen Meerschweinchen eine grössere Glykogenausbeute in der Leber durch Anwendung von Glukose mit Cebion als durch Zucker allein. Hirsch[4] konnte auch zeigen, dass schon Cebion allein bei normalen Tieren eine Vermehrung des Leberglykogens verursachen kann. Watanabé[5] bestätigte in dieser Klinik mit normalen Kaninchen den Befund von Altenburger und fand weiter einen Wiederaufbau des durch Leberschädigungen verloren gegangenen Glykogenvorrates bei Applikation der l-Ascorbinsäure.

Über das nährere Wesen der Vitamin C-Wirkung auf den Glykogenbestand sind wir aber zur Zeit noch wenig unterrichtet. Es fragt sich zunächst, in welcher Beziehung denn die Glykogenwirkung des Vitamins C zur antiskorbutischen steht. Man muss also klarstellen, ob jene nur auf die antiskorbutisch wirksamen Substanzen beschränkt ist, oder noch weit über die nahe verwandten Stoffe, die antiskorbutisch nicht immer so charakterisiert sind, verbreitet vorkommt. Manche Pflanzenstoffe sind bekanntlich antiskorbutisch stark wirksam und die Frage liegt so nahe, ob derartige Substanzen auch wirklich mit der Glykogenwirkung einhergehen, und wenn dies der Fall sei, weiter, ob

letztere Wirkung mit deren Vitamin C-Gehalt parallel gehe. Um auf all diese Fragen Antwort zu geben, habe ich in dieser Arbeit ausser l-Ascorbinsäure, der bekannten synthetisch dargestellten C-Vitaminsubstanz, noch d-Araboascorbinsäure in Betracht gezogen, welche ein Isomer ersterer ist, aber nach Untersuchungen von Dalmer und Moll,[6] Demole[7] und Zilva[8] in antiskorbutischer Wirkung jener weit nachstehen soll. Auch standen verschiedene Pflanzenstoffe bei dieser Arbeit zur Verfügung, deren Wirkung auf den Zuckerstoffwechsel überhaupt noch wenig bekannt ist. Soweit mir zugänglich, gibt Collazo[9] als einzige Literatur an, dass Glukose mit Orangensaft den normalen Kaninchen in den Magen eingeführt einen grösseren Glykogengehalt der Leber verursacht als Glukose allein. Wie man im folgenden aus den Ergebnissen meiner Untersuchungen sehen soll, üben die Pfanzenstoffe in der Regel eine ausgeprägte Wirkung auf den Glykogenbestand aus. Die Wirkungen der Naturprodukte sind aber wegen ihrer komplizierten Zusammensetzungen im allgemeinen schwer zu analysieren. Deswegen habe ich zur Vergleichsuntersuchung noch einige chemisch dargestellte Flavonverbindungen herangezogen, die Herr Prof. Huzise am Chemischen Institut dieser Universität mir freundlichst überliess, wofür ich ihm an dieser Stelle meinen verbindlichsten Dank aussprechen möchte.

Die Flavonverbindungen stehen bekanntlich mit Vitamin P in inniger Beziehung, wie aus der Untersuchung von Szent-Györgyi[10] hervorgeht, der aus Zitronensaft Citrin, eine Flavonverbindung aus Hesperidin und Eryodictyolglykosid isolierte, das zusammen mit Ascorbinsäure eingebracht, die Entwicklung des Skorbutes verhüten soll.

II. Versuchsmethodik.

Die Versuchskaninchen wurden in der Regel 7 Tage oder noch länger mit bei uns allgemein gebräuchlicher Laboratoriumkost, die ausschliesslich aus Okara bestand, vorher ernährt.

Zur Bestimmung der in der Leber neugebildeten Glykogenmenge ist es sehr dienlich, das Vorratglykogen vorher vollständig aus dem Organ ausgeschwemmt zu haben, ohne aber dadurch das Parenchym allzusehr zu schädigen, und zu diesem Zweck ist unter anderen die von Nagao und Otomo[11] ausgearbeitete Lezithinmethode vorzuziehen, die darin besteht, dass man den Kaninchen pro kg 10 ccm 10%iger Lezithinaufschwemmung intravenös injiziert, wodurch innerhalb von 6 bis 24 Stunden danach ein vollständiger Glykogenschwund zustande kommt. In Anbetracht dessen habe ich zu meinem Versuche stets 12 Stunden vor der Einverleibung von Vitamin C oder den naheverwandten Substanzen, deren Wirkung auf Glykogenbildung zu untersuchen war, eine

Lezithininfusion genau nach der genannten Vorschrift ausgeführt, um ein klares Bild der neugebildeten Glykogenausbeute ohne Verwischen durch die präformierte Substanz zu gewinnen. Die Glykogenneubildung zu veranlassen, habe ich pro kg 10 ccm 25 g/dl Glukoselösung gleichzeitig mit dem Vitamin ebenfalls intravenös eingebracht, wodurch nach Nagao und Otomo[11)12)] 3 Stunden danach trotz der vorherigen Lezithininfusion eine ansehnliche Glykogenmenge in der Leber zur Beobachtung kommen soll.

Die Glykogenbestimmung geschah nach der in dieser Klinik modifizierten Bierry[13)] Yamakawaschen[14)] Methode,[15)] wobei zur Reduktionsprobe die nach Bertrand herangezogen wurde.

Zur Ermittlung des Vitamin C-Gehaltes der zur Untersuchung gekommenen Naturprodukte benutzte ich die von Fujita und Iwatake[16)] modifizierte Birch, Harris und Raysche[17)] Mikrobestimmungsmethode, bei welcher das von Tillmans[18)] zuerst eingeführte 2,6-Dichlorphenol-Indophenol immer gebraucht wurde. Dass der dadurch gefundene Wert mit der antiskorbutischen Wirkung gut in Einklang kommt und deswegen als Vitamin C-Gehalt anzusehen ist, wurde schon von Harris und Ray[19)] bestätigt.

Dass der Glykogenbestand der Kaninchenleber besonders bei der Glukosezufuhr je nach den Jahreszeiten sich ziemlich verschieden verhält, ist von Kimura[20)] in dieser Klinik eingehend studiert worden. Mit Rücksicht darauf habe ich unten die Ergebnisse der Versuche, in denen verschiedene Fruchtarten, die in verschiedenen Monaten reifen, zur Untersuchung kamen, je nach den Jahreszeiten, in welchen sie ausgeführt wurden, in 2 Reihen von den wärmeren und kälteren abgeteilt geschildert.

III. Versuchsergebnisse.

1. *Glykogenbildung aus eingeführtem Traubenzucker in den kälteren Jahreszeiten.*

Um den Kontrollwert für die aus infundierter Glukose in der Leber gebildete Glykogenmenge in den kälteren Monaten, die sich von Oktober bis März erstrecken, zu gewinnen, wurde ein Versuch vorausgeschickt, indem wie eingangs erörtert, die Kaninchen pro kg 10 ccm von 25 g/dl Zuckerlösung 12 Stunden nach der vorläufigen glykogenausschwemmenden Lezithininfusion intravenös erhielten und nach weiteren 3 Stunden abgetötet wurden. Die gefundene Zahl von 1,16% stellt die Glykogenmenge dar, die dabei aus eingeführter Glukose in der Leber neugebildet wurde.

Tabelle 1.

Glykogengehalt der Leber der Normalkaninchen nach Glukosezufuhr.

3 Std. nach intravenöser Infusion von 2,5 g Glukose.

(2.–3. Oktober 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
1	1940	1910	55	0,92
2	1750	1690	47	1,08
3	1710	1620	39	1,38
4	1810	1750	49	1,22
5	1620	1510	58	1,21
Durchschnitt				1,16

2. *Fördernde Wirkung der l-Ascorbinsäure auf die Glykogenbildung.*

In einem derartigen Versuch wie der vorige wurde gleichzeitig mit der Zuckerinfusion pro kg 1 ccm Redoxon, das 50 mg l-Ascorbinsäure als Natriumsalz enthält, subkutan eingespritzt. Die Glykogenausbeute betrug 1,90%, vermehrte sich also durch Ascorbinsäurezusatz um 63%, was die Beobachtung Watanabés[5] gewissermassen bestätigte.

Tabelle 2.

Glykogengehalt der Leber nach Zufuhr von l-Ascorbinsäure und Glukose.

3 Std. nach Zufuhr von 50 mg l-Ascorbinsäure und 2,5 g Glukose.

(5.–6. Oktober 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
6	2080	1940	49	1,69
7	1540	1360	44	1,92
8	1890	1700	55	1,50
9	2100	1930	47	2,24
10	2400	2060	63	1,86
Durchschnitt				1,90

3. *Fördernde Wirkung der d-Araboascorbinsäure auf die Glykogenbildung.*

In diesem Versuch wurde bei sonst gleicher Anordnung wie dem vorigen statt Redoxon dieselbe Menge Viton gebraucht, das pro ccm 50 mg d-Araboascorbinsäure enthält.

Tabelle 3.

Glykogengehalt der Leber nach Zufuhr von d-Araboascorbinsäure und Glukose.

3 Std. nach Zufuhr von 50 mg d-Araboascorbinsäure und 2,5 g Glukose.

(15.–16. Oktober 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
11	1930	1770	65	1,89
12	1520	1470	55	1,61
13	1620	1550	57	2,18
14	1770	1650	52	1,86
15	1760	1620	55	1,79
Durchschnitt				**1,87**

Das gefundene Leberglykogen war 1,87%, die um 61% den Kontrollwert überwog. Die d-Araboascorbinsäure wirkt demnach auf die Glykogenbildung fast ebenso stark fördernd wie die l-Ascorbinsäure, wenn sie auch, wie bekannt, antiskorbutisch viel schwächer wirksam als letztere ist.

4. *Wirkung einer Mischung von l-Ascorbinsäure und Glukose auf die Glykogenbildung.*

Um zu prüfen, ob l-Ascorbinsäure in Glukoselösung haltbar sei, wurden 50 mg der Substanz in 10 ccm einer 25 g/dl Glukoselösung gelöst, unter Luftabschluss sterilisiert und aufbewahrt. Die Lösung wurde in Menge von pro kg 10 ccm den Kaninchen intravenös injiziert, wodurch Leberglykogen von 1,68% verursacht wurde. Die Zahl ist ein bisschen kleiner als beim Versuch, bei dem die einzelnen Substanzen getrennt infundiert wurden (vgl. Vers. 2), übersteigt aber den Kontrollwert um 45%.

Tabelle 4.

Glykogengehalt der Leber nach Infusion einer Mischung von l-Ascorbinsäure und Glukose.

3 Std. nach Infusion von 50 mg l-Ascorbinsäure und 2,5 g Glukose.

(21.–22. Oktober 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
16	1770	1640	58	1,77
17	1940	1890	61	1,45
18	1560	1550	48	1,85
19	1730	1530	59	1,86
20	1630	1510	47	1,47
Durchschnitt				1,68

5. *Derselbe Versuch wie oben mit d-Araboascorbinsäure.*

In demselben Versuch wie oben wurden statt l-Ascorbinsäure 50 mg d-Araboascorbinsäure gebraucht, wobei eine Glykogenausbeute von 1,66% gefunden wurde, die ebenfalls etwas kleiner als beim entsprechenden Versuch getrennter Infusion, aber um 43% grösser als der Kontrollwert ist. Da in diesen zwei letzten Versuchen l-Ascorbinsäure und d-Araboascorbinsäure betreffs der Wirkung auf den Glykogenbestand sich als ganz gleich erwiesen, so wurde in den folgenden Versuchen ausschliesslich d-Araboascorbinsäure statt l-Ascorbinsäure gebraucht, denn wir müssen zur Zeit wegen der Kriegsnot teuere ausländische Patentwaren möglichst ausschliessen.

Tabelle 5.

Glykogengehalt der Leber nach Infusion einer Mischung von d-Araboascorbinsäure und Glukose.*

3 Std. nach Infusion von 50 mg d-Araboascorbinsäure und 2,5 g Glukose.

(24.–25. Oktober 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
21	1660	1550	41	1,80
22	1760	1630	65	1,48
23	1620	1410	45	1,89
24	1880	1580	55	1,45
25	2180	2030	56	1,69
Durchschnitt				1,66

6. *Wirkung des Zitronensaftes auf die Glykogenbildung (Citrus medica L. subsp. Limonum Hook).*

Zur Untersuchung wurde zunächst den Kaninchen eine Mischung von 10 ccm 25 g/dl Glukoselösung und 1 ccm frischen Zitronensaftes pro kg intravenös injiziert, wonach ein Leberglykogen von 2,17% gefunden wurde (Tab. 6). Daraus ergibt sich, dass die aus injiziertem Zucker neugebildete Glykogenausbeute durch Zusatz des Zitronensaftes um 90% zugenommen hatte und die Glykogenbildung fördernde Wirkung von nur 1 ccm Zitronensaftes noch stärker als die von 50 mg l-Ascorbinsäure ist.

* Eine derartige Mischung soll in den folgenden Tabellen kurz als „d-Araboascorbinsäure+Glukose" geschrieben werden.

Tabelle 6.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Fleischsaft der Zitrone.

3 Std. nach Infusion von Zitronensaft 1 ccm und Glukose 2,5 g.

(1.–2. November 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
26	1930	1750	52	1,74
27	1920	1690	56	2,52
28	1940	1850	58	2,24
29	1830	1740	47	2,28
30	2150	1930	58	2,06
Durchschnitt				2,17

Im zweiten Versuch wurde wieder pro kg 1 ccm Zitronensaftes der gleichen Mischung von Glukose und d-Araboascorbinsäure, die in Versuch 5 verwendet wurde, hinzugesetzt. Dadurch schnellte die Glykogenausbeute auf 3,06% (Tab. 7) empor, vermehrte sich also im Vergleich mit dem Versuch ohne Zitronensaftzusatz um 90% (vgl. Vers. 5). Der Vitamin C-Gehalt des gebrauchten Zitronensaftes betrug in reduzierender Form 47,6 mg % und in Gesamtmenge 48,8 mg %, also 1 ccm Saft, in welcher Menge er bei diesem Versuch zur Verfügung stand, enthält allenfalls 0,5 mg Vitamin, die ja, auch wenn sie als ganzes mit Ascorbinsäure gleichzustellen ist, bloss ein Hundertstel der in den obigen Versuchen (vgl. Vers. 2 und 4) gebrauchten Menge derselben Substanz ausmacht. Von diesem Gesichtspunkt aus muss man zur

Tabelle 7.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit frischem Zitronensaft.

3 Std. nach Infusion von 1 ccm Zitronensaft, 50 mg d-Araboascorbinsäure und 2,5 g Glukose.

(2.–3. November 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
31	1860	1730	59	3,98
32	1740	1590	53	4,03
33	1520	1480	48	2,44
34	1790	1540	50	1,88
35	1630	1420	48	2,95
Durchschnitt				3,06

Erklärung der beobachteten unübertrefflichen Wirkung des frischen Zitronensaftes zu Gunsten der Glykogenneubildung die Anwesenheit eines anderen noch unbekannten Stoffes ausser Ascorbinsäure annehmen. Man kann natürlich Zweifel hegen, ob die reduzierenden Substanzen besonders die Zuckerarten, die in Zitronensaft enthalten sind und als Quelle der Glykogenneubildung zur Verfügung stehen können, dabei zur Vermehrung der Glykogenausbeute beigetragen haben. Ein derartiger Mehrbetrag der Glykogenausbeute, der auch wirklich bestünde, kann aber ohne weiteres ignoriert werden, wenn man daran denkt, dass die Menge der reduzierenden Substanzen in 1 ccm Zitronensaftes, die sich aus meiner Bestimmung nur einem Traubenzucker von 0,05 g entsprechend ergab, beim Arbeiten mit Infusion einer pro kg 2,5 g Zuckermenge gar nicht in Betracht kommt.

Im dritten Versuch wurde weiter die Eigenschaft des oben erwähnten neuen Stoffes untersucht, ob er in aktivem Zustand in Alkohol übergehen könne. Dazu wurde 1 Volum frischen Zitronensaftes mit 5 Volumen 95%igen Alkohols gemischt, kalt aufbewahrt und nach 2 Wochen filtriert. Das Filtrat wurde unter vermindertem Druck bei 60°C auf 1/3 Volum eingeengt. Der so hergestellte Extrakt entspricht also einer zweifachen Verdünnung des frischen Zitronensaftes. 2 ccm derartigen Extraktes wurden zusammen mit 10 ccm der gleichen Mischung von Glukose und d-Araboascorbinsäure, die in Versuch 5 gebraucht wurde, den Kaninchen intravenös injiziert, wodurch eine Glykogenausbeute von 2,47% resultierte (Tab. 8). Diese Zahl ist kleiner als beim Versuch mit frischem Zitronensaft, doch um 50% grösser im Vergleich mit dem Versuch ohne Zitronensaftzusatz. Daraus geht

Tabelle 8.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Alkoholextrakt des Zitronensaftes.

3 Std. nach Infusion. Alkoholextrakt des Zitronensaftes 2 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(15.-16. November 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
36	1880	1730	50	2,12
37	1920	1890	60	1,90
38	1610	1470	63	1,66
39	2220	1910	74	3,92
40	2210	1900	69	2,77
Durchschnitt				2,47

hervor, dass der in Rede stehende Stoff des Zitronensaftes, der auf die Glykogenbildung fördernd wirkt, in Alkohol übergeht und ziemlich gut haltbar ist.

7. *Versuch mit Saft der Mandarine (Citrus aurantium L. subsp. nobilis Makino).*

Bei Anwendung von frischem Mandarinensaft und Glukose unter sonst gleichen Anordnungen wie in den vorigen Versuchen fand man Leberglykogen von 2,02% (Tab. 9), das aber in einem anderen Versuch durch Zusatz der d-Araboascorbinsäure sich auf 2,43% vermehrte (Tab. 10). Die Glykogenbildung aus infundiertem Zucker gewinnt demnach durch Zusatz des frischen Mandarinensaftes 45 resp. 80%

Tabelle 9.

Glykogengehalt der Leber nach Infusion von Glukose und frischem Fleischsaft der Mandarine.

3 Std. nach Infusion. Fleischsaft der Mandarine 1 ccm, Glukose 2,5 g.

(7.–8. November 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
41	1590	1450	55	1,87
42	1900	1750	62	2,27
43	1730	1560	55	1,77
44	1710	1540	50	2,31
45	1650	1530	52	1,90
Durchschnitt				2,02

Tabelle 10.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit frischem Mandarinensaft.

3 Std. nach Infusion. Mandarinensaft 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(8.–9. November 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
46	1680	1570	67	2,44
47	1700	1600	45	2,61
48	1530	1480	55	2,08
49	1600	1460	52	2,28
50	1610	1540	56	2,76
Durchschnitt				2,43

mehr Ausbeute. Durch Anwendung eines Alkoholextraktes statt frischen Mandarinensaftes sank das Leberglykogen der Kaninchen, die gleichzeitig mit d-Araboascorbinsäure und Glukose behandelt wurden, auf 2,24% (Tab. 11), die doch um 35% grösser im Vergleich zum Versuch ohne Extraktzusatz sind (vgl. Vers. 4). Der Vitamin C-Gehalt des Mandarinensaftes betrug 29,4 mg % als reduzierende Form und 33,6% in totaler Menge.

Tabelle 11.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Alkoholextrakt des Mandarinensaftes.

3 Std. nach Infusion. Alkoholextrakt des Mandarinensaftes 2 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(22–23. November 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
51	1700	1660	57	1,91
52	1760	1680	62	2,99
53	1720	1690	57	2,21
54	1630	1440	59	1,68
55	1500	1430	45	2,41
Durchschnitt				2,24

8. *Versuch mit Saft der bitteren Orange (Citrus aurantium L. subsp. amara Engl.).*

Bei gleichzeitiger Zufuhr des frischen Saftes der bitteren Orange betrug die aus infundiertem Zucker gebildete Glykogenmenge in der Leber 1,60%, war also um 27% grösser im Vergleich mit dem Kontrollwert (Tab. 12). Bei Anwendung der d-Araboascorbinsäure war

Tabelle 12.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Fleischsaft der bitteren Orange.

3 Std. nach Infusion. Fleischsaft der bitteren Orange 1 ccm, Glukose 2,5 g.

(1.–2. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
56	1680	1580	55	1,62
57	1450	1370	44	1,54
58	1660	1520	52	1,48
59	1710	1540	50	1,64
60	1710	1570	55	1,72
Durchschnitt				1,60

das Leberglykogen durch Zusammenwirken des frischen Saftes 2,12% (Tab. 13), durch Alkoholextrakt statt frischen Saftes 1,99% (Tab. 14). Der Saft der bitteren Orange war demnach als Förderer der Glykogenbildung etwas weniger wirksam als der Zitronen- und Mandarinensaft. Als der Vitamin C-Gehalt des gebrauchten Saftes liess sich die reduzierende Form von 24,4 mg % und die totale Menge von 25,5 mg % bestimmen.

Tabelle 13.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure+ Glukose" mit frischem Saft der bitteren Orange.

3 Std. nach Infusion. Fleischsaft der bitteren Orange 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(2.–3. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
61	1870	1780	60	1,88
62	1680	1550	67	2,02
63	1590	1520	47	2,54
64	1730	1630	54	2,17
65	1740	1540	55	1,97
Durchschnitt				2,12

Tabelle 14.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure+ Glukose" mit Alkoholextrakt des Saftes der bitteren Orange.

3 Std. nach Infusion. Alkoholextrakt des Saftes der bitteren Orange 2 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(15.–16. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
66	1900	1770	69	2,28
67	1810	1700	58	1,97
68	1840	1650	59	1,82
69	1660	1520	55	2,09
70	1710	1640	52	1,77
Durchschnitt				1,99

9. *Versuch mit Pompelmuse (Citrus Sabon Sieb.).*

Durch Infusion von frischem Fleischsaft der Pompelmuse und Glukose wurde Leberglykogen von 1,73% gewonnen, das um 50% grösser war als der Kontrollwert (Tab. 15). Durch Hinzufügen der

d-Araboascorbinsäure zum Infusionsmittel erhöhte sich die Glykogenausbeute noch auf 1,97% (Tab. 16), bei Anwendung des Alkoholextraktes statt frischen Saftes sank sie wieder auf 1,81% (Tab. 17). In einer zweiten Versuchsreihe stand der Pressaft der Pompelmusschale statt des Fleischsaftes zur Verfügung. Durch Beimischen von pro kg 1 ccm desselben Pressaftes mit zu infundierender Glukoselösung fand man Leberglykogen von 1,79% (Tab. 18), das fast ebenso gross wie beim Versuch mit Fleischsaft war. Die gleichzeitige Infusion der d-Araboascorbinsäure vermehrte die Glykogenausbeute auf 2,05% (Tab. 19), das Ersetzen des frischen Saftes durch Alkoholextrakt in sonst gleichem Versuch setzte das Leberglykogen auf 1,85% herab (Tab. 20). Der Vitamin C-Gehalt des Fleischsaftes betrug 35,7 mg % in reduzierender Form und 37,5 mg % in toto, der des Schalensaftes 66,7 mg %

Tabelle 15.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Fleischsaft der Pompelmuse.

3 Std. nach Infusion. Fleischsaft der Pompelmuse 1 ccm, Glukose 2,5 g.

(8.–9. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
71	1750	1610	62	1,68
72	1730	1640	62	1,50
73	1860	1780	64	1,89
74	1660	1580	52	1,84
75	1630	1520	48	1,76
Durchschnitt				1,73

Tabelle 16.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit frischem Fleischsaft der Pompelmuse.

3 Std. nach Infusion. Fleischsaft der Pompelmuse 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(9.–10. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
76	1760	1710	75	2,12
77	1720	1630	60	2,24
78	1750	1720	72	1,82
79	1970	1860	67	1,81
80	1830	1790	60	1,84
Durchschnitt				1,97

Tabelle 17.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure+ Glukose" mit Alkoholextrakt des Pompelmusfleischsaftes.

3 Std. nach Infusion. Alkoholextrakt des Pompelmusfleischsaftes 2 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(22.–23. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
81	1540	1440	45	1,77
82	1510	1500	45	2,00
83	1570	1470	52	2,06
84	1590	1460	50	1,54
85	1720	1620	54	1,66
Durchschnitt				1,81

Tabelle 18.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Pompelmusschalensaft.

3 Std. nach Infusion. Pompelmusschalensaft 1 ccm, Glukose 2,5 g.

(25.–26. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
86	1650	1630	60	2,00
87	1740	1690	47	1,58
88	1520	1480	57	1,70
89	1690	1640	87	1,92
90	1420	1370	42	1,75
Durchschnitt				1,79

Tabelle 19.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure+ Glukose" mit Pompelmusschalensaft.

3 Std. nach Infusion. Pompelmusschalensaft 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(26.–27. Dezember 1937)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
91	1850	1650	60	1,78
92	1580	1520	44	2,12
93	1700	1580	65	2,00
94	1540	1400	49	1,97
95	1760	1620	59	2,40
Durchschnitt				2,05

Tabelle 20.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure+ Glukose" mit Alkoholextrakt des Pompelmusschalensaftes.

3 Std. nach Infusion. Alkoholextrakt des Pompelmusschalensaftes 2 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(9.–10. Januar 1938).

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
96	1820	1570	62	2,01
97	1720	1620	57	1,86
98	1670	1550	55	2,13
99	1480	1470	45	1,75
100	1540	1430	50	1,50
Durchschnitt				1,85

resp. 67,2 mg %. Bekanntlich ist bei Pompelmuse die Schale viel vitaminreicher als das Fleisch und in der Tat erwies sich nach meiner Bestimmung erstere als ungefähr 2 fach so gross als letztere, trotzdem sind betreffs der Glykogenbildung fördernden Wirkung die beiden Bestandteile fast gleich stark, woraus schon hervorgeht, dass die in Rede stehende glykogenanreichernde Wirkung mit dem Vitamin C-Gehalt nicht immer parallel geht.

10. Versuch mit Apfelsaft.

Frischer Apfelsaft zur Glukoselösung beigemischt und infundiert, verursachte bei den Kaninchen einen Glykogengehalt der Leber von 1,73% (Tab. 21). Hinzusetzen der d-Araboascorbinsäure zum Infusionsmittel erhöhte die Ausbeute auf 2,38% (Tab. 22), die aber durch Ersetzen des frischen Saftes durch dessen Alkoholextrakt auf 2,17% sank (Tab. 23).

Der Apfel hat sich nach Untersuchungen von Holst und Frölich[21] gegen experimentellen Skorbut als wenig wirksam erwiesen. Damit in Einklang stehen die Beobachtungen von Tillmans[22] und Fujita,[23] dass der Vitamin C-Gehalt des Apfels überhaupt sehr gering ist. Bei meiner Untersuchung fand ich fast keine reduzierende Form, wohl aber eine totale Menge von 5,3 mg %, die ja ungefähr 1/9 der des Zitronensaftes und 1/5 der des bitteren Orangensaftes entsprechen. Trotzdem überwiegt der Apfel betreffs der glykogenanreichernden Wirkung die bittere Orange, wenn er auch der Zitrone etwas nachsteht. Man kann auch an dieser Stelle sehen, dass die Wirkung der

Tabelle 21.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Apfelsaft.

3 Std. nach Infusion. Apfelsaft 1 ccm, Glukose 2,5 g.

(15.–16. Januar 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
101	2360	2180	70	1,96
102	1710	1540	57	1,72
103	1630	1450	47	1,78
104	1390	1330	40	1,63
105	1700	1640	58	1,56
Durchschnitt				1,73

Tabelle 22.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit frischem Apfelsaft.

3 Std. nach Infusion. Apfelsaft 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(15.–16. Januar 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
106	1530	1360	45	2,51
107	1620	1520	50	2,42
108	1640	1600	49	2,66
109	1810	1690	72	2,08
110	1730	1570	47	2,25
Durchschnitt				2,38

Tabelle 23.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Alkoholextrakt des Apfelsaftes.

3 Std. nach Infusion. Alkoholextrakt des Apfels 2 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(30.–31. Januar 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
111	1420	1380	39	2,35
112	1630	1570	67	1,85
113	1530	1430	51	2,56
114	1450	1270	43	1,98
115	1690	1620	62	2,09
Durchschnitt				2,17

Obste auf den Glykogenbestand keineswegs mit ihrem Vitamingehalt parallel läuft.

11. *Versuch mit Grünteeinfusum.*

5 g Grüntee (Thea sinensis L.) wurden mit 100 ccm Wasser bei 50°C 30 Minuten lang digeriert und filtriert. 1 ccm des Filtrates pro kg mit der üblichen Menge von Glukose zur Infusion gebraucht, wurde das Leberglykogen zu 1,71% (Tab. 24), bei Addieren der d-Araboascorbinsäure dazu das zu 1,89% gefunden (Tab. 25).

Dass der Grüntee sehr C-vitaminreich ist, wurde schon von Miura und Tuzimura[24] bestätigt. Miura[25] hat auch darauf hingewiesen, dass eine zu heisse Auslaugung Verlust des Vitamins verursacht. Da ich bei meiner Untersuchung die reduzierende Vitaminform

Tabelle 24.

Glykogengehalt der Leber nach Infusion von Glukose mit Grünteeinfusum.

3 Std. nach Infusion. Grünteeinfusum 1 ccm, Glukose 2,5 g.

(4.–5. Februar 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
116	1990	1780	44	1,72
117	2050	1770	60	2,01
118	1550	1460	48	1,86
119	1630	1520	55	1,37
120	2040	1960	59	1,57
Durchschnitt				1,71

Tabelle 25.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Grünteeinfusum.

3 Std. nach Infusion. Grünteeinfusum 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(5.–6. Februar 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
121	1640	1530	55	1,74
122	1850	1710	52	1,85
123	1890	1760	54	2,19
124	1840	1680	42	1,78
125	1950	1850	59	1,88
Durchschnitt				1,89

% von 250 mg % und Gesamtmenge von 278,6 mg % in Grünteesubstanz und 13,5 mg % resp. 15,6 mg % in 20 fach verdünntem Infusum fand, so ist klar, dass die ganze Vitaminmenge dabei ausgelaugt wurde. Betreffs der Glykogenanreicherung ist das Grünteeinfusum im Gegensatz zu seinem reichen Vitamingehalt verhältnismässig weniger wirksam.

12. *Wirkungen der Flavonverbindungen auf die Glykogenbildung.*

Zur Untersuchung kamen Flavonverbindungen wie l-Matteucinol, Homoeryodictyol, Desmethoxymatteucinol und 7-Oxyflavanon, die zu Flavanongruppe gehören. l-Matteucinol in Menge von 2 mg pro kg wurde in 1 ccm 95%igen Alkohols gelöst und mit 10 ccm von 25 g/dl Glukoselösung gemischt, den Kaninchen intravenös injiziert, wodurch eine Glykogenausbeute von 1,49% entstand (Tab. 26). Diese Zahl ist nur um 28% grösser als der Kontrollwert mit Zuckerinfusion allein. Durch Addieren der d-Araboascorbinsäure dazu wurde die Ausbeute auf 2,47% gesteigert (Tab. 27), die um 48% grösser als der ohne l-Matteucinol gefundene Wert ist.

Unter einer ähnlichen Versuchsanordnung wurde eine Glykogenausbeute von 1,35% bei Anwendung von Homoeryodictyol und Glukose (Tab. 28) und eine von 1,82% bei Zusammenwirken der d-Araboascorbinsäure (Tab. 29) gefunden.

Homoeryodictyol förderte demnach die Glykogenbildung nicht deutlich nachweisbar. Etwas wirksamer waren indessen zwei andere

Tabelle 26.

Glykogengehalt der Leber nach Infusion von Glukose mit l-Matteucinol.

3 Std. nach Infusion. l-Matteucinol 2 mg, Glukose 2,5 g. (12.–13. Februar 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
126	1640	1520	45	1,33
127	1890	1820	61	1,28
128	1650	1630	66	1,78
129	1570	1520	49	1,38
130	1630	1440	59	1,68
Durchschnitt				1,49

Tabelle 27.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit l-Matteucinol.

3 Std. nach Infusion. l-Matteucinol 2 mg, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(22.–23. Februar 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
131	1680	1510	52	1,91
132	1620	1470	47	2,80
133	2130	1850	74	2,68
134	1640	1470	65	2,02
135	1440	1320	47	2,93
Durchschnitt				**2,47**

Tabelle 28.

Glykogengehalt der Leber nach Infusion von Glukose mit Homoeryodictyol.

3 Std. nach Infusion. Homoeryodictyol 2 mg, Glukose 2,5 g.

(1.–2. März 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
136	1650	1560	52	1,44
137	1680	1500	55	1,26
138	1750	1620	48	1,66
139	1510	1400	40	1,17
140	1750	1700	54	1,22
Durchschnitt				**1,35**

Tabelle 29.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Homoeryodictyol.

3 Std. nach Infusion. Homoeryodictyol 2 mg, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(4.–5. März 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
141	1840	1660	57	1,58
142	1890	1600	56	2,10
143	1530	1420	43	1,66
144	1630	1530	61	1,86
145	1620	1450	60	1,92
Durchschnitt				**1,82**

Flavonverbindungen. Desmethoxymatteucinol und Glukose verursachten 1,55% Glykogen (Tab. 30), durch Addieren der d-Araboascorbinsäure sogar 2,03% (Tab. 31). 7-Oxyflavanon und Glukose erbrachten 1,54% Glykogenausbeute (Tab. 32), die sich durch Zusammenwirken der d-Araboascorbinsäure auf 2,25% steigerte (Tab. 33). Betreffs der glykogenanreichernden Wirkung kommen unter 4 Flavonverbindungen, die zur Untersuchung herangezogen wurden, l-Matteucinol und 7-Oxyflavanon an der Spitze, dann Desmethoxymatteucinol und zuletzt Homoeryodictyol.

Tabelle 30.

Glykogengehalt der Leber nach Infusion von Glukose mit Desmethoxymatteucinol.

3 Std. nach Infusion. Desmethoxymatteucinol 2 mg, Glukose 2,5 g.

(8.–9. März 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
146	1660	1580	57	1,39
147	1860	1760	67	1,20
148	1830	1700	57	1,73
149	1430	1370	50	1,90
150	1370	1850	42	1,54
Durchschnitt				1,55

Tabelle 31.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Desmethoxymatteucinol.

3 Std. nach Infusion. Desmethoxymatteucinol 2 mg, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(11.–12. März 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
151	1850	1700	58	1,50
152	1800	1510	54	2,05
153	1630	1520	45	1,33
154	1410	1320	41	2,41
155	1950	1790	62	2,88
Durchschnitt				2,03

Tabelle 32.

Glykogengehalt der Leber nach Infusion von Glukose mit 7-Oxyflavanon.

3 Std. nach Infusion. 7-Oxyflavanon 2 mg, Glukose 2,5 g.

(14.–15. März 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
156	1660	1650	50	1,36
157	1500	1400	47	1,45
158	1710	1670	65	1,61
159	1430	1420	40	1,80
160	1610	1570	57	1,50
Durchschnitt				1,54

Tabelle 33.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit 7-Oxyflavanon.

3 Std. nach Infusion. 7-Oxyflavanon 2 mg, d-Araboascorbinsäure 50 mg Glukose 2,5 g.

(20.–21. März 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
161	1510	1400	48	2,92
162	1910	1840	56	1,90
163	1610	1380	55	2,91
164	1700	1530	56	1,84
165	1620	1400	47	1,68
Durchschnitt				2,25

13. Kontrollwerte des Glykogenbestandes der Kaninchen für wärmere Jahreszeiten.

Da in den folgenden Versuchen die Früchte, die in wärmeren Jahreszeiten reifen, zur Verfügung standen, so mussten die Kontrollwerte des Glykogenbestandes der Tiere für diese Zeiten von neuem untersucht werden.

Aus einem in Juni ausgeführten Versuch geht hervor, dass nach Infusion der üblichen Menge von Glukose durchschnittlich 0,98% Glykogen in der Leber gefunden wurden. Diese Zahl ist im Vergleich mit dem schon erwähnten Oktoberversuch um 17% kleiner.

In einem zweiten, ebenso in Juni ausgeführten Versuch, bei dem Glukose zusammen mit d-Araboascorbinsäure verwendet wurde, fand

man eine Glykogenausbeute von 1,47% (Tab. 35), die im Vergleich mit dem Oktoberversuch um 13% vermindert ist. Durch Addieren der d-Araboascorbinsäure gewinnt man aber eine um 50% grössere Ausbeute.

Tabelle 34.

Glykogengehalt der Leber nach Infusion von Glukose.

3 Std. nach Infusion von 2,5 g Glukose.

(3.-4. Juni 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
166	1610	1520	48	1,12
167	1590	1540	45	0,95
168	1790	1620	57	0,88
Durchschnitt				0,98

Tabelle 35.

Glykogengehalt der Leber nach Infusion von „d-Aroboascorbinsäure + Glukose"

3 Std. nach Infusion. d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(6.-7. Juni 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
169	1650	1560	60	1,44
170	1830	1730	48	1,88
171	1730	1550	44	1,58
Durchschnitt				1,47

14. *Versuch mit Sommerorangensaft (Citrus aurantium L. subsp. Natsudaidai, Hayata).*

Bei Anwendung von Glukose mit 1 ccm Sommerorangensaft fand man Leberglykogen von 1,25% (Tab. 36), bei Addieren der d-Araboascorbinsäure dazu das von 1,83% (Tab. 37), das sich aber durch Ersetzen des Fleischsaftes mit Pressaft der Fruchthülle auf 2,36% steigerte (Tab. 38). Der Schalensaft ist demnach auf die Glykogendepots wirksamer als der Fleischsaft. Jener enthielt 55,6 mg % von reduzierender Form in gesamter Vitamin C-Menge von 59,1 mg %, während bei diesem 20,8 mg % resp. 23,8 mg % gefunden wurden. Die Wirkung der beiden Gehäusebestandteile auf den Glykogenbestand scheint demnach

dem Vitamingehalt zu entsprechen; das ist aber wahrscheinlich als ein Zufallsbefund anzusehen.

Tabelle 36.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Fleischsaft der Sommerorange.

3 Std. nach Infusion. Fleischsaft der Sommerorange 1 ccm, Glukose 2,5 g.

(10.–11. Juni 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
172	1510	1350	47	1,24
173	1690	1580	50	1,18
174	1620	1510	49	1,32
Durchschnitt				1,25

Tabelle 37.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit frischem Fleischsaft der Sommerorange.

3 Std. nach Infusion. Fleischsaft der Sommerorange 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(10.–11. Juni 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
175	1630	1520	58	1,74
176	1690	1430	53	2,08
177	1460	1360	40	1,68
Durchschnitt				1,83

Tabelle 38.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit frischem Saft der Sommerorangeschale.

3 Std. nach Infusion. Saft der Sommerorangeschale 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(10.–11. Juni 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
178	1600	1530	44	1,89
179	1730	1640	57	2,20
180	1650	1520	48	3,00
Durchschnitt				2,36

15. *Versuch mit Erdbeerensaft.*

Die übliche Glukosemenge mit 1 ccm frischen Erdbeerensaftes rief eine Glykogenausbeute von 1,33% hervor (Tab. 39), Zusatz der d-Araboascorbinsäure dazu erhöhte letztere auf 2,09% (Tab. 40).

Der Erdbeerensaft ist bekanntlich sehr C-vitaminreich und es ergab sich aus meiner Bestimmung die reduzierende Form von 62 mg %, in Gesamtmenge von 75 mg %.

Tabelle 39.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Erdbeerensaft.

3 Std. nach Infusion. Erdbeerensaft 1 ccm, Glukose 2,5 g.

(20.–21. Juni 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
181	1820	1730	47	1,34
182	1740	1630	52	1,38
183	1500	1320	40	1,27
Durchschnitt				1,33

Tabelle 40.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure+ Glukose" mit frischem Erdbeerensaft.

3 Std. nach Infusion. Erdbeerensaft 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(20.–21. Juni 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
184	2210	2070	57	1,96
185	2070	2060	55	2,31
186	1980	1840	50	2,01
Durchschnitt				2,09

16. *Versuch mit Tomatensaft.*

Glukose mit frischem Tomatensaft von 1 ccm gemischt und injiziert, verursachte einen Glykogengehalt der Leber von 1,46% (Tab. 41), beim Hinzusetzen der d-Araboascorbinsäure fand man einen von 2,26% (Tab. 42), durch Gebrauch des Alkoholextraktes statt frischen Fruchtsaftes sank die Glykogenausbeute auf 1,71% (Tab. 43). Aus der Vita-

mingehaltbestimmung ergaben sich 17,9 mg % in reduzierender Form und 21 mg % als Gesamtmenge. Diese Zahlen sind noch kleiner als ein Drittel des Vitamingehaltes des Erdbeerensaftes, trotz der stärkeren Einwirkung auf den Glykogenbestand.

Tabelle 41.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Tomatensaft.

3 Std. nach Infusion. Tomatensaft 1 ccm, Glukose 2,5 g.

(13.–14. Juli 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
187	1590	1700	44	1,52
188	1560	1410	41	1,45
189	1560	1440	40	1,40
Durchschnitt				1,46

Tabelle 42.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Tomatensaft.

3 Std. nach Infusion. Tomatensaft 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(13.–14. Juli 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
190	1810	1700	53	2,18
191	1770	1670	54	2,43
192	2110	1960	60	2,16
Durchschnitt				2,26

Tabelle 43.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit Alkoholextrakt des Tomatensaftes.

3 Std. nach Infusion. Alkoholextrakt des Tomatensaftes 2 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(29.–30. Juli 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
193	1650	1570	47	1,79
194	1620	1480	50	1,72
195	1580	1510	49	1,62
Durchschnitt				1,71

17. *Versuch mit Wassermelonensaft (Citrullus vulgaris Schrad.).*

Eine Mischung von Glukose und 1 ccm frischen Wassermelonensaftes brachte Leberglykogen von 1,52% hervor (Tab. 44), Addieren der d-Araboascorbinsäure vermehrte die Ausbeute auf 2,29% (Tab. 45). Die Vitamin C-Menge des Wassermelonensaftes betrug 3,6 mg % als reduzierende Form und 4,9 mg % als totale Menge. Der verhältnismässig vitaminarme Wassermelonensaft übertrifft aber bezüglich der Glykogenanreicherung den Erdbeerensaft, der an Vitamingehalt ca. 15 fach grösser ist.

Tabelle 44.

Glykogengehalt der Leber nach Infusion von Glukose mit frischem Wassermelonensaft.

3 Std. nach Infusion. Wassermelonensaft 1 ccm, Glukose 2,5 g.

(3.–4. August 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
196	1520	1490	52	1,26
197	1650	1600	47	1,56
198	1590	1500	42	1,74
Durchschnitt				1,52

Tabelle 45.

Glykogengehalt der Leber nach Infusion von „d-Araboascorbinsäure + Glukose" mit frischem Wassermelonensaft.

3 Std. nach Infusion. Wassermelonensaft 1 ccm, d-Araboascorbinsäure 50 mg, Glukose 2,5 g.

(3.–4. August 1938)

Kaninchen-Nr.	Körpergewicht (g)		Gewicht der Leber (g)	Leberglykogen (%)
	Anfangs	am Ende		
199	1810	1670	50	1,63
200	1610	1500	60	2,70
201	1680	1580	49	2,55
Durchschnitt				2,29

IV. Besprechung.

Durch vorangehende Versuche wurde der von Altenburger[3] und Watanabé[5] zuerst bemerkte Befund völlig bestätigt, dass die

l-Ascorbinsäure die Glykogenbildung aus zugeführtem Zucker auch bei den nicht skorbutischen Tieren fördert. Die Träger dieser glykogenanreichernden Wirkung sind aber nach meiner Untersuchung nicht allein auf die l-Ascorbinsäure beschränkt, sondern kommen unter den verwandten chemischen Substanzen, und sogar auch noch unter den Naturprodukten des Pflanzenreiches weit verbreitet vor.

Unter ersteren Substanzen kommt vor allem die d-Araboascorbinsäure in Betracht, die nach Untersuchungen der vorangehenden Autoren betreffs der antiskorbutischen Wirkung viel schwächer als l-Ascorbinsäure sein soll. Dieser Isomer der l-Ascorbinsäure erwies sich für den Glykogenbestand der Leber als ebenso stark wirksam wie l-Ascorbinsäure. Schon daraus kann man annehmen, dass die glykogenanreichernde Wirkung dieser Substanzen mit der antiskorbutischen nicht identisch ist. Wenn man die Definition von Vitamin C gang und gäbe auf einen antiskorbutisch wirksamen Stoff beschränkt, so wird die auf den Glykogenbestand beziehende Wirkung natürlich in der des betreffenden Vitamins nicht miteinbegriffen. Daher nenne ich bei dieser Darstellung die in Frage stehende Erscheinung vorläufig Vitamin C_3-Wirkung—da der Name C_2 schon von Euler[26] dem antipneumonischen Faktor, der in Zitronensaft vorkommen soll, gegeben wurde—, wobei aber über die Anwesenheit der von dem eigentlichen Vitamin C oder besser Vitamin C_1 stofflich verschiedenen Substanzen nichts präjudiziert wird, denn wie man schon aus oben geschildertem sieht, können dieselben Substanzen wie l-Ascorbinsäure oder d-Araboascorbinsäure sowohl antiskorbutische, als auch glykogenanreichernde Wirkung ausüben.

Die Inkongruenz der antiskorbutischen und der Glykogenbildung fördernden Wirkung, d. h. der Vitamin C_1- und der Vitamin C_3-Wirkung an Stärke kann man schon bei diesen synthetisch dargestellten Substanzen beobachten. In dieser Arbeit wurde die Vitamin C_3-Wirkung ausserdem noch mit verschiedenen Pflanzenstoffen untersucht, wobei sie stets mehr oder minder deutlich nachweisbar war; ihre Stärke aber sehr oft mit den Vitamin C-Gehalten, die durch die von Tillmans[18] eingeführte Dichlorphenol-Indophenolmethode ermittelt wurden, nicht im Einklang stand. Ein Beispiel kann vielleicht dieses Sachverhältnis völlig klarstellen. Wie schon erwähnt kann man bei Zuckerinfusion durch Addieren von 1 ccm frischen Zitronensaftes mehr Glykogenausbeute erzielen als durch das der l-Ascorbinsäure von 50 mg, welch letztere nach Untersuchung von Watanabé[5] eine optimal wirksame Menge darstellt. Da aber 1 ccm Zitronensaft nur ca. 0,5 mg Vitamin C in sich enthält, so besteht kein Zweifel, dass die

Glykogenbildung fördernde Wirkung des Zitronensaftes nicht allein von seinem Vitamin C-Gehalt abhängt, sondern man muss auch annehmen, dass ein spezifischer Faktor in den pflanzlichen Naturprodukten existiert, der sicher von der uns bekannten Ascorbinsäure verschieden ist und auf die Glykogenbildung über hundertmal so stark wie letztere anregend wirkt.

Die Wirkungen der Pflanzenstoffe auf den Glykogenbestand sind indessen ziemlich kompliziert, worauf ich noch später zurückkomme. Hier sei noch die Erwägung der einschlägigen Wirkungen der einfachen chemischen Substanzen vorausgeschickt. Wie bekannt wurde in den letzten Zeiten von Szent-Györgyi[10] aus dem Zitronensaft eine Art Flavonglykosid isoliert, das wegen seiner Wirkung auf die Permeabilität der Gefässe vom Autor Vitamin P zu nennen vorgeschlagen wurde. Ob dieser neue Stoff eine Wirkung auf den Glykogenbestand auszuüben vermag, darüber sind wir heute noch nicht unterrichtet. Jedenfalls kommen die Flavonglykoside in der Pflanzenwelt weit verbreitet vor.

Für meine Untersuchung kamen unter den Flavonverbindungen l-Matteucinol, Homoeryodictyol, Desmethoxymatteucinol und 7-Oxyflavanon in Betracht. Durch Anwendung von Glukose + l-Matteucinol gewann man um 0,33% mehr Glykogen als die Menge bei der von Zukker allein, woraus dieser Betrag auf die direkte l-Matteucinolwirkung zurückzuführen ist. Bei Anwendung von Glukose + d-Araboascorbinsäure + l-Matteucinol fand man ferner 2,47% Glykogen (vgl. Vers. 12), das noch um 0,48% grösser ist als die berechnete Summe der Zahl von 1,66%, die sich aus Versuch 4 mit Glukose + d-Araboascorbinsäure ergab, und der von 0,33%, die wie oben erwähnt die Ausbeute der direkten l-Matteucinolwirkung bei der Zuckerinfusion darstellt. Diesen Mehrbetrag von 0,48% kann man vielleicht als die Folge der zwischen l-Matteucinol und d-Araboascorbinsäure bestehenden Wechselbeziehung ansehen, wodurch ihre Wirkungen einseitig oder gegenseitig verstärkt werden. Ein derartiger Mehrbetrag von Glykogen, der die berechnete Summe übertrifft, wurde auch bei Anwendung von Glukose + d-Araboacorbinsäure + 7-Oxyflavanon gefunden, wenn er auch nicht mehr als 0,21% betrug. Beim Versuch mit Homoeryodictyol oder Desmethoxymatteucinol kann man auch eine direkte Förderung der Glykogenbildung mehr oder minder deutlich nachweisen, wohl aber keine wechselseitige Verstärkung der Wirkungen zwischen diesen Substanzen und d-Araboascorbinsäure, denn die gefundene Glykogenausbeute bei gleichzeitiger Anwendung dieser Substanzen stimmte stets

mit der berechneten Summe der aus einzelnen Proben hervorgehobenen Werte gut überein. Somit gelangen wir zum Schluss, dass die vier Arten Flavonverbindungen, die zur Untersuchung herangezogen wurden, alle die Glykogenbildung aus infundiertem Zucker direkt fördern, was aber die Eigenschaften betrifft, die Wirkung der d-Araboascorbinsäure auf die Glykogenbildung zu verstärken, so lässt sie sich nur bei zwei von ihnen nachweisen.

Demnach kann man schon in den Wirkungen der chemischen Flavonverbindungen auf den Glykogenbestand zwei Faktoren erkennen, nämlich einen, der die Glykogenbildung direkt fördert (Faktor I) und einen zweiten, der die betreffende fördernde Wirkung der anderen Substanzen verstärkt (Faktor II). Derartige Wirkungen können natürlich auch die Naturprodukte der Pflanzenwelt beherbergen, in denen die Glykoside vorkommen, die Flavonverbindungen als Agrykon in sich enthalten. Um diese Frage zu veranschaulichen, komme ich wieder darauf zurück, die einzelnen Ergebnisse der Versuche mit Pflanzenstoffen näher in Betracht zu ziehen.

Bei Anwendung von 1 ccm Zitronensaft mit Zuckerlösung fand man Leberglykogen von 2,17% (vgl. Tab. 6), die 1,01% grösser sind als der Wert beim Versuch ohne Zitronensaft. Dieser Mehrbetrag ist also auf die Wirkung des Faktors I zurückzuführen. Beim Versuch mit Zitronensaft + Zucker + d-Araboascorbinsäure kam eine Glykogenausbeute von 3,06% zur Beobachtung, die 0,39% grösser ist als die berechnete Summe von 1,66% aus Zucker + d-Araboascorbinsäure und 1,01% aus der direkten Wirkung des Zitronensaftes. Diese Differenz zwischen der gefundenen und berechneten Glykogenmenge muss man als Folge der durch Faktor II verstärkten Araboascorbinsäurewirkung ansehen. Auf diese Weise sind bei Zitronensaft 2 Faktoren von Vitamin C nachzuweisen.

Aus einer derartigen Berechnungsweise gewinnt man bei Erdbeere als Wirkung des Faktors I 0,35% und als die des Faktors II 0,27%, bei Tomate 0,48% resp. 0,31% und bei Wassermelone 0,54% resp. 0,28%, welche alle demnach auch zur Kategorie gehören, die beide Faktoren von Vitamin C_3 tragen.

Etwas abweichend verhielt sich aber die andere Gruppe der Pflanzenstoffe, die zur Untersuchung kam. Bei diesen liess sich auch die Anwesenheit des Faktors I stets nachweisen, aber der Fakter II wurde vermisst, oder kam nur undeutlich zur Beobachtung. Z. B. fand man bei Apfelsaft 0,57% Glykogen als Wirkung des Faktors I. Die beim Versuch mit Apfelsaft + d-Araboascorbinsäure + Zucker gefundene

Glykogenausbeute betrug 2,38%, die nur ein bisschen grösser ist als die berechnete Summe von 2,23%.

Beim Versuch mit Apfelsaft kann man als Wirkung des Faktors II eine kleine, aber noch positive Zahl ausrechnen. Bei den übrigen Pflanzenstoffen hingegen gewinnt man aber eine immer mehr oder minder grosse negative Zahl, nämlich bei Mandarinensaft lässt sich als Wirkung des Faktors I eine so grosse Glykogenausbeute wie 0,86% nachweisen, wozu im Gegensatz die des Faktors II mit −0,09% steht. Bei bitterer Orange, Pompelmuse, Sommerorange und Grüntee kamen ebenfalls nur negative Zahlen für Faktor II zur Beobachtung, wenn auch Faktor I eine mehr oder minder reichliche Glykogenausbeute verursachte.

Die verschiedenen Naturprodukte der Pflanzenwelt lösen demnach gleich wie chemische Flavonverbindungen teils nur eine direkte Bereicherung des Depotsglykogens aus, teils aber verstärken sie ausserdem die glykogenbereichernde Wirkung der anderen Stoffe. Erst durch Annahme der komplizierten Vorgänge kann man die Beobachtung erklären, dass eine Menge frischen Zitronensaftes betreffs der Glykogenanreicherung die Wirkung einer nach dem Masse des Vitamin C-Gehaltes 100 mal so grossen l-Ascorbinsäure übertrifft, welche letztere die reine C-Vitaminsubstanz repräsentiert.

Die Ergebnisse der Vitamin C-Bestimmung deckten sich im allgemeinen gut mit denen der vorangehenden Autoren. Die verschiedenen Orangearten sind an C-Vitamin reich und besonders deren Schalen reicher als das Fleisch. Soweit es die Orangearten betrifft, wurde die Glykogenausbeute entsprechend ihrem Vitamin C-Gehalt vermehrt gefunden, woraus man aber auf eine völlige Abhängigkeit der Glykogenwirkung vom Vitamingehalt nicht schliessen darf, denn diese beiden gehen bei Versuchen mit anderen Fruchtarten ziemlich auseinander. Apfel und Wassermelone, die sehr C vitaminarm sind, übertreffen in ihrer Glykogenwirkung den C-reichen Erdbeerensaft bei weitem. Diese Beobachtung zusammen mit den anderen schon geschilderten Ergebnissen, die ebenfalls auf die Inkongruenz des Vitamingehaltes mit der Glykogenwirkung hinweisen, führt uns ungezwungen zur Annahme von der Existenz anderer Faktoren in den Pflanzenstoffen, die neben l-Ascorbinsäure die Glykogenbereicherung verursachen. Näheres über diese Faktoren und besonders, ob sie mit dem von Szent-Györgyi[10] beschriebenen P-Vitamin in Beziehung stehen, harrt noch weiterer Klärung.

V. Schluss.

1. l-Ascorbinsäure fördert die Glykogenbildung in der Leber, mit welcher Wirkung zwar von gleicher Stärke auch ihr Isomer d-Araboascorbinsäure einhergeht.

2. Eine dergleiche direkte Glykogenwirkung findet man auch bei den verschiedenen Pflanzenstoffen, wie Früchten und Grüntee, deren Stärke aber dabei nicht mit dem Vitamin C-Gehalt parallel geht. Eine innige Beziehung der glykogenbereichernden Wirkung zum Vitamin C ist auch deshalb weniger wahrscheinlich, weil eine kleine Menge Pflanzenstoffe, welche nach dem Mass des Vitamin C-Gehaltes einem Mehrzehntel oder sogar einem Hundertstel der optimal wirkenden l-Ascorbinsäure entsprechen, gewöhnlich eine so kolossale Glykogenausbeute verursacht, dass die durch letztere auslösbare Menge noch weit übertrifft.

3. Die Pflanzenstoffe tragen ausserdem noch andere Faktoren, die die glykogenanreichernde Wirkung andere Substanzen, z. B. der l-Ascorbinsäure oder d-Araboascorbinsäure, stark anregen, wodurch die Glykogenausbeute noch weiter vermehrt wird. Mit dieser indirekten Wirkung ist aber nicht jeder Pflanzenstoff versehen, im Gegensatz zur direkten Wirkung, die weit verbreitet unter den pflanzlichen Naturprodukten vorkommt.

Die ähnlichen Wirkungen wie die Pflanzenstoffe auf den Glykogenbestand, und zumal eine direkte und eine indirekte, kommen auch bei den chemisch dargestellten Flavonverbindungen zur Beobachtung, wobei aber die indirekte Wirkung ebenfalls nur bei einem Teile auftritt.

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The Epinephrine Potentiating Effect of Sodium Ascorbate in Allergy

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The striking success of antihistaminic agents such as pyribenzamine, benadryl and several others in controling the nasal turgescence and rhinorrhea of seasonal hay fever, and in relieving the symptoms of urticaria, led to exaggerated hopes of similar results in the treatment of asthma. While the effectiveness of antihistaminic agents has lent considerable support to the role of histamine in allergy, it may also tend to overemphasize the histamine side.

In asthma pyribenzamine apparently fails to dilate the bronchioles thus giving little or no comfort to the patient. This would suggest that normal bronchiolar tone is the result of an active balance between the constricting action of histamine and the dilating capacity of epinephrin.

In a previous publication I[1] described the experimental microscopic observation of bronchiolar reaction to histamine and the antihistamine effect of Vitamin C. In this experiment the lung of the white New Zealand rabbit was filled with gelatin and then excised and kept viable in Ringer-Locke-Glucose solution. After chilling, the lung was cut so that each section contained a bronchiole. This was mounted on a small ring so that it could be observed under the microscope. The gelatin was now washed out of the lung tissue and the whole preparation was maintained viable in the Ringer-Locke-Glucose solution at a fixed temperature. When histamine was added to the solution, the bronchiole promptly contracted and the change of size was drawn through the camera lucida and calculated by means of the planimeter. After the addition of Vitamin C, the bronchiole recovered from the contractions and became moderately dilated. If epinephrine was added the dilatation would increase further. [For the exact technique reference may be made to the original article.]

At the time of the reporting of this experiment I interpreted the dilatation of the bronchiole after the histamine contraction as attributable to the antihistamine action of Vitamin C. In the light of subsequent study I am inclined to believe that the counteraction of the histamine contraction was due, not to the chemical neutralization of histamine, but rather to the potentiation of the epinephrin already present in the viable section of bronchiole. This explanation became even more probable from the demonstration that, if additional epinephrin was added, the bronchiole dilated to twice the size of the normal bronchiole before the histamine had been added, thus showing that a very active dilator effect was produced.

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As one followed this hypothesis it seemed important to study every possible avenue for potentiating the body reserves of epinephrine present in the tissues of the asthmatic. In this investigation it became apparent that the whole suprarenal mechanism was involved in an intimate relationship with Vitamin C and Sodium. The clinical administration of sodium ascorbate was uniquely effective.

In practical application it was possible to demonstrate that daily dosages between 1000 mg. to 2000 mg. of Vitamin C afforded many patients considerable relief from allergic manifestations and some even striking and dramatic improvement. There remained, however, a group of patients that was either not benefited or only moderately improved.

Analysis of these latter patients showed, as a rule, concomitant infection with a history of repeated colds or bronchial infections. They were of the type usually classified as persons with a lowered resistance to infection.

When, as a result of technical advance in Vitamin C research by the author, there was made commercially available the dry sodium salt of Vitamin C, an investigation of its use in allergy was undertaken. Originally, it was believed that the chief advantage of the sodium salt would be in the fact that it was a neutral form of Vitamin C. Since the high dosage of Vitamin C entailed a daily amount of 1.5 grams to 2 grams, gastric irritation was experienced frequently. Another factor was the continued, and, at times, undesirable increase in ingested acid.

While the kidney has the power to form ammonia for the purpose of neutralizing an acid urine, it may also produce acid as NaH_2PO_4 as well as free organic acid, provided the Ph does not fall much lower than 4.0. When, however, large amounts of acid are introduced into or produced in the body, the ammonia producing mechanism of the kidney cannot suffice and the sodium bicarbonate of the blood is depleted for the purpose of neutralizing the acid. The CO_2 is exhaled by the lungs and the sodium salt of the acid appears in the urine; hydrochloric acid, for example, is excreted as sodium chloride, sulphuric acid as sodium sulphate.

If acid be fed and base is withheld from the diet for some days, sodium salts are excreted at first and a little extra potassium, calcium or magnesium appears. Gradually, as the requirement for base becomes more insistent, abnormally large amounts of potassium are excreted. The body manifestly resists potassium loss as long as possible; probably because potassium and magnesium are essential to cell function in a more direct way than sodium. The depression of sodium levels may play an important role in kidney blockage from drugs of the sulfa group. Thus the availability of sodium ascorbate gave us an important agent in acid base equilibrium.

Analysis of blood and tissues shows a remarkable difference in distribution of cations —according to Holmes.[2]

Cation	Milligrams in 100 grams of plasma	Milligrams in 100 grams of muscle
Sodium	138.0	60.0
Potassium	18.0	360.0
Calcium	10.5	10.0
Magnesium	2.7	23.0

It is apparent that tissue cells contain far more potassium than sodium and that sodium loss would come first. Sodium, however plays a vital role in connection with the normal metabolism of the adrenal cortex. One could consider that the adrenal cortical hormone, sodium and Vitamin C bear a recripocal relationship to each other. It is, therefore, not surprising to find that the cortical hormone protected against histamine shock just as Vitamin C does. Thus Ingle[3] showed the protective effect of fragments of viable cortical tissue and of transplants of cortical tissue on the resistance of suprarenalectomized rats to histamine shock. These observations were confirmed by Wyman[4] and Tum Suden.[5] They also showed that the increased susceptibility of the rat to histamine after suprarenalectomy depends on the loss both of medullary and cortical function. In an earlier paper on Histamine-Adrenalin Balance in Allergy I[1] pointed out that shock of the most varied sort ranging from severe burns and trauma to poisonings have a common histamine reaction directly analogous to nasal allergy and asthma.

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ypothesis that this triad of substances may lay a basic role in allergy and that the deficiency of any one or more of these elements would predispose to allergic reactions. The Pottengers[6] had already treated a group of 50 allergic children suffering with asthma by means of whole adrenal gland and a high salt intake. In addition a diet rich in vitamins and minerals was given. Improvement was observed in 84 per cent of the patients.

It now remained to observe the isolated effect of sodium ascorbate. Marine & Baumann[7] had found that daily intraperitoneal injections of various sodium salts such as isotonic sodium chloride, sodium acetate, Ringers Solution, prolonged the lives of suprarenalectomized controls. They pointed out the specific value of sodium salts in maintaining the life of the animal. Similar findings were observed by Stewart and Rogoff,[8] although they found that salt solution could not be used as a substitute for cortical hormone. Loeb and his co-workers[9] extended the studies and from their work suggested that suprarenal insufficiency was associated with a primary loss of sodium through the kidney. Harrop and his associates[10] were able to maintain bilaterally suprarenalectomized dogs for as long a period as five months without the use of any suprarenal gland preparation or extract by the administration of sodium chloride and sodium bicarbonate alone. Withdrawal of the salt then produced typical suprarenal insufficiency. It has been shown by Zwemer and Truszkowski[11] that a drop in sodium concentration in the blood is associated with a significant rise in the Potassium concentration. The production of such an electrolyte disturbance, as suggested by Gilman and Yannet,[12] decreases the natural resistance of unoperated rats to histamine poisoning (Perla and Sandberg[13]).

However, Perla and Marmorstan[14] found that, when cortin is administered during the early period of insufficiency, the resistance of suprarenalectomized animals is raised to a striking degree, whereas when epinephrin is similarly administered, the resistance is raised only to a slight degree. By the administration of cortin, they were able to raise the resistance of suprarenalectomized rats to histamine almost to the normal level.

A further interesting point arose when the cortical hormone was used in experimental infections. Scott, Bradford, Hartman and McCoy[15] determined the effect of the cortical hormone on the resistance of normal animals. Three types of intoxication were used; diphtheria toxin in the guinea pig, trypanosome equiperdum infection in the rat and pneumococcus infection in the mouse. No protection was obtained against one minimum lethal dose of toxin by administration of cortical extract in amounts of one and one half cc. Injections of cortin were begun after the injection of diphtheria toxin. Similar results were produced in the other infections. All the mice were dead within 32 hours. The administration of cortical extract did not produce any appreciable survival period of white mice following infection with pneumococcus.

Zemmer & Jungeblut[16] demonstrated some protection by the injection of cortical extract in normal guinea pigs against a minimum lethal dose of diphtheria toxin. On the whole the results were not striking, although apparently, under certain conditions, the administration of a large dose of a cortical extract may protect the guinea pig against more than one M.L.D. of diphtheria toxin.

Thaddea,[17] however, observed definitely beneficial effects of injection of cortical hormone when combined with Vitamin C on the resistance of guinea pigs to diphtheria. Cosentino,[18] likewise, reported favorable results in rabbits infected intraperitoneally with cultures of staphylococcus aureus by the use of Vitamin C and adrenal cortical hormone.

Heuer and Andrus[19] have reported that cortical extract has a protective action against the shock produced by intravenous injection of intestinal loop fluid into dogs. Meek[20] also noted beneficial effects from the administration of cortin to dogs suffering from shock following intestinal distension. There is the further observation of Wolfram and Zwemer[21] in the amelioration of anaphylactic shock in guinea pigs by the prior administration of cortin.

The interrelationship of histamine sensi-

tivity to adrenal cortical insufficiency and sodium and Vitamin C led to the hypothesis that nasal allergy and asthma may be precipitated by an imbalance of the triad cortical hormone-Sodium-Vitamin C. In view of the reciprocal capacity that exists between them, a study was undertaken on the effect of sodium ascorbate, particularly in those allergic patients who were somewhat refractory to high dosage ascorbic acid. The beneficial effects were strikingly greater than ascorbic acid itself. There was, in addition, marked freedom from gastric irritation with the use of 2 grams daily. Increased diuresis was

	Name	Age	Sex	History	Treatment and Results
1.	E. H.	64	F.	Sneezing for about 2 years in summer and early spring. Some asthmatic attacks during this time. Some food allergies. Nasal passage always feels congested.	4/21—Started taking sodium ascorbate tablets 3 t.i.d. Sneezing at this time. 4/28—Relieved completely by sodium ascorbate tablets of sneezing. No congestion of nose, continued taking 3 t.i.d. 5/24—Relieved completely of all symptoms, no congestion of nose. Mucosa normal, no irritation of stomach. Slight increase of urine during 1st 2 weeks, the following week no increase due to taking of aspirin for extraction of tooth. Now taking 1 sodium ascorbate tablet t.i.d.
2.	B. W.	25	F.	Hay fever for past 15 years. More severe last two seasons. Unable to work during hay fever season.	Allergic testing 4+ reaction to ragweed and timothy. Started hay fever injections 5/12/45. Given 1 every 5 days. Taking sodium ascorbate pills, 2 t.i.d. Series of injections finished 7/2/45. Patient not seen again at office. Mother reports that patient had a very good summer. Very little sneezing and running of nose. No stomach irritation. Considers this the best summer she has had for a number of years.
3.	L. G.	45	M.	Sneezing from March 20 to May 14 every year. Has nose bleed during sneezing attacks. Allergic testing shows no sensitivity to scratch tests. Itching of eyes and nose also at this time.	Allergy testing for spring grasses and trees shows no specific reaction. Given special spring tree vaccine, starting 12/18/44. First symptoms of spring grass fever on 3/5/45. Started sodium ascorbate pills, 1 t.i.d. On 3/26/45 given 2 sodium ascorbate pills, t.i.d. Slept the first time in 2 weeks. 3/27 taking 3 sodium ascorbate pills, t.i.d. On 3/28 patient states that he feels like an entirely different person with very little sneezing after taking the sodium ascorbate tablets. 4/14 patient reports very little sneezing, 40 oz. urine excreted in 24 hours. 4/18 no sneezing, no running of nose for last 2 weeks. Sleeps well, feeling excellent. 4/25 nose congested. Patient without sodium ascorbate pills for one week. Sneezing some. Given sodium ascorbate pills, 2 t.i.d. 5/2 nose not congested. No sneezing, no running.
4.	R. B.	30	M.	Hay fever and asthma for 20 years. 4+ reaction to dust, feathers, ragweed and timothy.	Started hay fever injections 5/14/45 combined with sodium ascorbate tablets, 3 t.i.d. Given hay fever injections twice weekly. Patient seen at office after Sept. 1 and stated that so far he has had the best season. After June 15, he took 2 sodium ascorbate tablets, t.i.d. Very little sneezing. No irritation of stomach or increase of urination.
5.	J. C.	22	F.	Hay fever for about 8-10 years. Suffers from asthmatic attacks during hay fever season. An uncle died of asthma.	Allergy testing showed 4+ to dust, ragweed and timothy. Allergic to foods as well. Given adrenalin injection to control hives and itching after testing for ragweed and timothy. Eyes swollen also. Started hay fever injections 5/7/45. Given 1 injection every 5 days. Started sodium ascorbate tablets, 3 t.i.d., at the same time as hay fever injections. Finished hay fever injections 6/28/45, completing series. At this time she was taking 2 sodium ascorbate tablets, t.i.d. Patient seen again 9/6/45, stating that at that time she had been entirely free of sneezing up to this time. Sneezing and symptoms usually worse from the end of Aug. to Sept. 15. Seen again 9/11 and 9/15. No symptoms at all at this time. No irritation of stomach, no increase of urination or diarrhea during the time the tablets were taken. Patient states that this is the first season she has been entirely free of any hay fever symptoms. No asthmatic attacks during season.
6.	B. B.	16	F.	Hay fever for 2 years. Sneezing and running nose. Sneezing starting in July through middle of August. No food allergies.	4+ reaction to ragweed and timothy. Started sodium ascorbate tablets, 1 t.i.d. on 6/13/44. Increased to 2 t.i.d., 7/1/45. No sneezing or running of nose at present. Patient returned to office 9/11/45. At that time patient stated that she went without pills one day and her nose started to run immediately. After taking 2 sodium ascorbate tablets nose stopped almost instantly. Slight heartburn at times. May be due to sodium ascorbate tablets. No increase of urination.

	Name	Age	Sex
7.	S. R.	35	F.
8.	P. S.	45	M.
9.	F. S.	27	F.
10.	L. G.	25	F.
11.	R. R.	6	M.
12.	N. H.	25	F.
13.	W. L.	46	M.

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	Name	Age	Sex	History	Treatment and Results
7.	S. R.	35	F.	Sneezing and running nose for last year with no relief. Hay fever for 17 years. Asthmatic attacks in hay fever season.	Allergy testing shows 4+ reaction to dust, ragweed and timothy, also allergic to some foods. Started hay fever injections 5/7/45. Given 1 injection every 5 days. Started sodium ascorbate pills, 3 t.i.d., at same time. Finished series of hay fever injections 6/28/45. Now taking sodium ascorbate tablets, 2 t.i.d. Patient seen again each week from 9/6/45 to 10/19/45. During this time patient reports entire relief from all symptoms of hay fever that she suffered during previous years. Has had no asthmatic attacks during the season. No irritation of stomach, slight increase in urination, no diarrhea. Sneezing and running of nose entirely cleared. Patient considers this the best season she has had for some time.
8.	P. S.	45	M.	Hay fever for last 7 years, asthmatic attacks at times. No food allergies.	Allergy testing: 4+ to ragweed and timothy. Started hay fever injections 5/3/45. Started sodium ascorbate tablets, 2 t.i.d., at the same time. Given injections every 5 days. Series of injections ending 6/29/45. Continuing to take sodium ascorbate tablets, 2 t.i.d. Returned to office 10/13/45. At this time patient states that he has had a much better season than previously. This is the first season that he has not had any asthmatic attacks. Very little sneezing. No irritation of stomach or increase in urination.
9.	F. S.	27	F.	Hay fever for last 8 years. Nasal stuffiness, sneezing and running. Had 6 injections of hydrochloric acid for hay fever without results. No asthmatic attacks. No known food allergies.	Allergic testing 4+ to timothy and ragweed. Started hay fever injections 5/10/45. Started sodium ascorbate, 2 t.i.d., at the same time. Series of injections finished 6/28/45. Patient visited office 9/3/45. Has had first hay fever symptom, but very little sneezing. Increased sodium ascorbate tablets to 3 t.i.d. 9/15 patient reports very little sneezing. Feeling better this year than at any previous time. No irritation of stomach, no increase of urination.
10.	L. G.	25	F.	History of hay fever in August since childhood.	8/15/45 started sodium ascorbate tablets, 2 t.i.d. Gastric upset after taking 2 t.i.d. for 2 days. 8/22/45 gastric upset, started taking 1 t.i.d. 9/4/45—No sneezing this season. Patient reports the best season since she can remember. During this season patient also took hay fever injections from local physician.
11.	R. R.	6	M.	Had hives at age of 3 with spots on chest. No nasal symptoms. At 4 developed sneezing and rhinitis in August and September.	Allergy testing shows child to be allergic to ragweed and timothy, also to most foods. 6/17/45—Started sodium ascorbate pills, 1 t.i.d. Continued with no sneezing or hives through entire hay fever season. Last season patient started on C.B. pills, 1 t.i.d., which caused upset stomach. Patient was then given nucleic acid with some relief but not as dramatic as the sodium ascorbate pills.
12.	N. H.	25	F.	Has had hay fever for the last 4 years, complicated by asthma the last 2 years. Allergy tests 3 years ago shows sensitivity to dust, ragweed and timothy.	5/24/45—Started ragweed injections 1 every 5. Also started taking sodium ascorbate tablets, 2 t.i.d. 6/15/45—Taking sodium ascorbate tablets, 3 t.i.d. No gastric disturbances. 6/27/45—Finished hay fever series. 9/10/45—Patient reported at office stating that attacks have been lessened this summer. When patient had an occasional severe attack accompanied by asthmatic attack, she took 3 sodium ascorbate tablets and attack was greatly relieved.
13.	W. L.	46	M.	Hay fever for 30 years with asthmatic attacks.	5/18/45—Started hay fever series 1 every 5 days. Also started sodium ascorbate pills, 3 t.i.d. 6/30/45—Slight digestive disturbance. Reduced dosage 2 t.i.d 9/5/45—Patient seen at office. Reports very little sneezing. Usually at this time patient suffers with hay fever. Mostly from 9/1 to 9/10. Taking 2 t.i.d. 9/12/45—Patient states that he had no sneezing at all this season.

noted almost as a rule. Several of the patients were intractable asthmatics who had been refractory to almost all forms of therapy, but these patients have remained free from asthma in a fairly continuous level of sodium ascorbate. The seasonal hay fever patients did particularly well.

In reviewing the results obtained with ascorbic acid, calcium ascorbate, and sodium ascorbate, it would appear that the inorganic ion plays a very important role. It is not improbable that many factors influence histamine sensitivity. Among these are enzymatic reactions and toxic factors that are favorably influenced by calcium ion, whereas another important group may be strikingly helped by the sodium ion. In both instances the inorganic ion, as the salt of ascorbic

acid, represents a very desirable form of the therapy in allergy.

The use of the sodium salt of ascorbic acid may also play an important role in other conditions such as urinary lithiasis, when the solvent action of the ascorbate on carbonates is utilized, as well as the solubilizing effect of the sodium ion.

While the desirability of a sodium salt of ascorbic acid was obvious, great technical obstacles presented themselves. There was no dry sodium salt of ascorbic acid available. The instability of ascorbic acid in alkali solution was well known and described in virtually every test dealing with ascorbic acid. In fact Sherman[22] points out the ready destruction of ascorbic acid by alkali, and the Merck Index also describes the same. When ascorbic acid decomposes, it goes to oxalic acid with its strong tendency to deplete the calcium ion. An unstable sodium salt of ascorbic acid would thus be less desirable than ascorbic acid itself. Karrer[23] had tried to prepare a sodium salt of ascorbic acid by reacting sodium ethylate in ethyl alcohol but found the procedure tricky and uncertain with a strong tendency to decomposition. It thus appeared that the preparation of a stable salt of sodium ascorbate was doomed to failure. Many different attempts to secure this valuable product were made unsuccessfully until one day the reaction was run between sodium methylate and ascorbic acid in methyl alcohol. This yielded a remarkably stable sodium ascorbate that was even more stable than Vitamin C itself and resisted heat in the oven for over 500 hours assuring freedom from such decomposition products as oxalic acid.

The explanation of this phenomenon led to much chemical speculation as to whether the sodium ion reacts with the hydroxyl in the second or third carbon atom, since the ring is not considered broken in the formation of the salt. Study of the reaction conducted by Karrer,[23] and compared to that which I carried out, showed a marked difference in the character of the crystallization which is apparently responsible for the differences in degree of stability. Thanks to the availability of the pure product the clinical investigation could be conducted.

CONCLUSIONS

1. The availability of the sodium salt of the ascorbic acid for oral use represents a marked advance in the therapeutic approach to allergy.

2. The increased sensitivity to histamine in suprarenalectomized animals suggests a relationship between adrenal cortical hormone-sodium-Vitamin C and allergy.

3. In refractory cases of allergy and asthma, sodium ascorbate was more effective than ascorbic acid.

32 East 67th Street.

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Radiation Therapy for Prevention of Deafness and Treatment of Upper Respiratory Tract Infections

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It is often difficult to determine the onset of impairment of hearing in childhood. Suspected patients should be given hearing tests, and in those subjects in whom a loss of hearing is found, radiation therapy should be instituted. In many children examined by me, the loss of hearing was recognized first by the parents. While most of these children had had their tonsils and adenoids removed, the majority still had hypertrophied lymphoid tissue present in the nasopharynx.

It frequently happens that following a tonsillectomy and adenoidectomy, symptoms of repeated colds and nasal infection, recurring otitis media, cough, and bronchitis will persist in some persons despite ordinary treatment. In these patients otolaryngological examination will generally disclose masses of residual tissue in the nasopharynx, especially around the eustachian tube orifices. This tissue cannot be removed by adenoidectomy as it is inaccessible to surgical extirpation. Any attempt at removal would traumatize the fossae of Rosenmüler resulting in cicatrical tissue and contraction of the mouth of the eustachian tubes.

Lymphoid tissue is known to be radiosensitive. The rays act on the germinal centers of the cells, inhibit mitosis and prevent further function and growth of new lymphocytes to replace those destroyed. In this way the masses of radiated tissue shrink and disappear. No further recurrence takes place.

It has been established definitely by Crowe, Burnham, Baylor, and their associates at the Johns Hopkins Hospital that radiation therapy will alleviate obstructive deafness starting in childhood as the result of excessive hypertrophied lymphoid tissue around the eustachian tube orifices in the nasopharynx. In fact, they claim as the result of this new therapy being applied during childhood that the number of patients with obstructive deafness should be reduced to 50 per cent in adults.

Examination of the ears of these children usually shows retracted drums, especially marked over Schrapnel's membrane, and some loss of normal lustre. There is an occasional pinkish periphery, or congested drums. Loss of hearing is found for both speech frequencies and for the high tones, the latter being the first impairment noted in most instances.

Other conditions which have been helped by the use of radium therapy are recurrent chronic middle ear infections; repeated nasal colds and postnasal discharge; continuous mouth breathing; and long continued aural discharge following paracentesis. Bronchial asthma and upper respiratory infections are further indications for this therapy, according to more recent reports.

Radon gas or the radium element itself can be used in a special Monell metal applicator 15 mm. in length, with an inside diameter of 1.7 mm. and a wall thickness of 0.3 mm. This is sufficient to absorb the caustic rays and to permit proper filtration of the therapeutic Beta and Gamma rays. A small chamber is designed at the end of the applicator which contains either 50 mg. of radium element or its equivalent in Radon gas.

After each nasal passage is anesthetized, under guidance of a nasopharyngoscope the

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Sodium Ascorbate in the Treatment of Allergic Disturbances

The Role of the Adrenal Cortical Hormone-Sodium-Vitamin C

by
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FOLLOWING AN EARLIER WORK on high dosage Vitamin C in allergy, it became apparent that a very significant role was played by the inorganic ion sodium when used as the salt of Vitamin C. By a detailed microscopic study of bronchiolar reactions it was demonstrated that Vitamin C had a definite antihistamine effect, or an epinephrine potentiality effect. In practical application it was possible to demonstrate that daily dosages between 1000 mg. to 2000 mg. of Vitamin C afforded in many cases considerable relief from allergic manifestations and in some cases even striking and dramatic improvement. There remained however a group of cases that were either not benefited or were only moderately improved.

Analysis of these latter cases showed as a rule concomitant infection with a history of repeated colds or bronchial infection. They were of the type usually classified as individuals with a lowered resistance to infection.

When, as a result of my technical advance in Vitamin C research there was made commercially available the dry sodium salt of Vitamin C an investigation of its use in allergy was undertaken. Originally, it was believed that the chief advantage of the sodium salt would be in the fact that it was a neutral form of Vitamin C, since the high dosage of Vitamin C entailed a daily amount of 1.5 gram to 2 grams, and gastric irritation was experienced frequently. Another factor was the continued and at times undesirable increase in ingested acid.

While the kidney has the power to form ammonia for the purpose of neutralizing an acid urine, it can also produce acid as $Na\ H_2PO_4$ and also as free organic acid, provided the pH does not fall much lower than 4.0. When, however, large amounts of acid are introduced into or produced in the body the ammonia producing mechanism of the kidney cannot suffice and the sodium bicarbonate of the blood is depleted for the purpose of neutralizing the acid. The CO_2 is exhaled by the lungs and the sodium salt of the acid appears in the urine; hydrochloric acid, for example, is excreted as sodium chloride, sulphuric acid as sodium sulphate.

If acid be fed and base is withheld from the diet for some days, sodium salts are excreted at first and little extra potassium, calcium or magnesium appears. Gradually, as the requirement for base becomes more insistent abnormally large amounts of potassium are excreted. The body manifestly resists potassium loss as long as possible: probably because potassium and magnesium are essential to cell function in a more direct way than sodium. The depression of sodium levels may play an important role in kidney blockage from drugs of the sulfa group. Thus the availability of sodium ascorbate gave us an important agent in acid base equilibrium.

Analysis of blood and tissues shows a remarkable difference in distribution of cations — according to Holmes.

Cation	*mgs. in 100 gms. of plasma*	*mgs. in 100 gms. of muscle*
Sodium	138.0	[illegible]
Potassium	18.0	36[illegible]
Calcium	10.5	1[illegible]
Magnesium	2.7	2[illegible]

It is apparent that tissue cells contain far more potassium than sodium and that sodium loss would come first.

Sodium, however, plays a vital role in connection with the normal metabolism of the adrenal cortex. One could consider that the adrenal cortical hormone, sodium and Vitamin C, bear a reciprocal relationship to each other. It is, therefore, not surprising to find that the cortical hormone protected against histamine shock similar to that of Vitamin C. Thus Ingle showed the protective effect of fragments of viable cortical tissue and of transplants of cortical tissue on the resistance of suprarenalectomized rats to histamine shock. These observations were confirmed by Wyman and Tum Suden. They also showed that the increased susceptibility of the rat to histamine after suprarenalectomy depends on the loss both of medullary and cortical function.

However, Perla and Marmorstan found that when cortin is administered during the early period of insufficiency, the resistance of suprarenalectomized animals is raised to a striking degree, whereas when epinephrin is, similarly administered, the resistance is raised only to a slight degree. By the administration of cortin, they were able to raise the resistance of suprarenalectomized rats to histamine almost to the normal level.

A further interesting point arose when the cortical hormone was used in experimental infections. Scott, Bradford, Hartman and McCoy determined the

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effect of the cortical hormone on the resistance of normal animals. Three types of intoxication were used; diphtheria toxin in the guinea pig, trypanosome equiperdum infection in the rat and pneumococcus infection in the mouse. No protection was obtained against one minimum lethal dose of toxin by administration of cortical extract in amounts of one and one-half cc. Injections of cortin were commenced after the injection of diphtheria toxin. Similar results were produced in the other infections. All the mice were dead within 32 hours. The administration of cortical extract did not produce any appreciable survival period of white mice following infection with pneumococcus.

Zwemer and Jungeblut showed some protection by the injection of cortical extract in normal guinea pigs against a minimum lethal dose of diphtheria toxin. On the whole the results were not striking although apparently under certain conditions the administration of a large dose of a cortical extract may protect the guinea pig against more than one M.L.D. of diphtheria toxin.

Thaddea, however, observed definitely beneficial effects of injection of cortical hormone when combined with Vitamin C on the resistance of guinea pigs to diphtheria. Cosentino likewise reported favorable results in rabbits infected intraperitoneally with cultures of staphylococcus aureus by the use of Vitamin C and adrenal cortical hormone.

Heuer and Andrus have reported that cortical extract has a protective action against the shock produced by intravenous injection of intestinal loop fluid into dogs. Meek also noted beneficial effects from the administration of cortin to dogs suffering from shock following intestinal distension. There is the further observation of Wolfram and Zwemer in the amelioration of anaphylactic shock in guinea pigs by the prior administration of cortin.

In an earlier paper on Histamine-Adrenalin Balance in Allergy, I pointed out that shock of the most varied sort ranging from severe burns and trauma to poisonings have a common histamine reaction directly analogous to nasal allergy and asthma.

The interrelationship of the adrenal cortical hormone to Vitamin C and Sodium led to the hypothesis that this triad of substances may play a basic role in allergy and that the deficiency of any one or more of these elements would predispose to allergic reactions. The Pottengers had already treated a group of 50 allergic children suffering with asthma by means of whole adrenal gland and a high salt intake. In addition a diet rich in vitamins and minerals was given. Improvement was observed in 84 per cent of the patients.

It now remained to observe the isolated effect of sodium ascorbate. Marine and Baumann had found that daily intraperitoneal injections of various sodium salts such as isotonic sodium chloride, sodium acetate, Ringers solution, prolonged the lives of suprarenalectomized rats to almost three times those of the untreated suprarenalectomized controls. They pointed out the specific value of sodium salts in maintaining the life of the animal. Similar findings were observed by Stewart and Rogoff, although they found that salt solution could not be used as a substitute for cortical hormone. Loeb and his coworkers extended the studies and from their work suggested that suprareral insufficiency was associated with a primary loss of sodium through the kidney. Harrop and his associates were able to maintain bilaterally suprarenalectomized dogs for as long a period as five months without the use of any suprarenal gland preparation or extract by the administration of sodium chloride and sodium bicarbonate alone. Withdrawal of the salt then produced typical suprarenal insufficiency. It has been shown by Zwemer and Truszkowski that a drop in sodium concentration in the blood is associated with a significant rise in the Potassium concentration. The production of such an electrolyte disturbance as suggested by Gilman and Yannet decreases the natural resistance of unoperated rats to histamine poisoning (Perla and Sandberg).

The interrelationship of histamine sensitivity to adrenal cortical insufficiency and sodium and Vitamin C led to the hypothesis that nasal allergy and asthma may be precipitated by an imbalance of the triad cortical hormone-Sodium-Vitamin C. In view of the reciprocal capacity that exists between them, a study was undertaken on the effect of sodium ascorbate, particularly in those allergic cases that were somewhat refractory to high dosage ascorbic acid. The beneficial effects were strikingly greater than ascorbic acid itself. There was in addition marked freedom from gastric irritation with the use of 2 grams daily. Increased diuresis was noted almost as a rule. Several of the cases were intractable asthmatics who had been refractory to almost all forms of therapy but have remained free from asthma in a fairly continuous level of sodium ascorbate. The seasonal hay fever cases did particularly well.

In reviewing the results obtained with ascorbic acid, calcium ascorbate and sodium ascorbate, it would appear that the inorganic ion plays a very important role. It would seem that many factors influence histamine sensitivity. Among these are enzymatic reactions and toxic factors that are favorably influenced by calcium ion whereas another important group may be related to adrenal cortical insufficiency and be strikingly helped by the sodium ion. In both cases the inorganic ion as the salt of ascorbic acid represent a very desirable form of therapy in allergy.

The use of the sodium salt of ascorbic acid may also play an important role in other conditions such as urinary lithiasis when the solvent action of the ascorbate on carbonates is utilized as well as the solubilizing effect of the sodium ion.

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CASE REPORTS ---- SODIUM ASCORBATE

NAME	Age	Sex	Diagnosis	History & Findings	Treatment & Results
1.G.H.	8	F	Asthma Food Allergy	At the age of 5 following an attack of measles, the child developed severe recurrent attacks of asthma, which recurred in a series of attacks every 5 to 6 months. The attacks were difficult to control and the patient was sent to Arizona where she remained for 2 years. While in Arizona her attacks were recurrent, but milder. The patient was brought to New York for further study. Nasal examination revealed bilateral ethmoiditis, associated with post nasal discharge and engorgement of the nasal mucosa. The blood picture showed: hemoglobin, 62%, color index, .6 with a slight leukocytosis and moderate eosinophilia.	The patient was placed on Sodascorbate therapy, 3 tablets 3 times a day. The patient was having asthmatic attacks at the time of examination. During the first week of therapy the attacks became much milder and ceased after the second week of therapy. She was on continuous Sodascorbate therapy 3 tablets 3 times daily for over a year with no recurrence of asthmatic attacks. The child gained in weight. The anemia was treated with twice weekly injections of Ironyl for the improvement of the blood picture. Tonsillectomy was also performed. Patient's mother's comment: "The child can now eat most anything without having an asthmatic attack."
2.L.G.	45	M	Nasal allergy, unknown origin, probably Spring trees	For 20 years has had attacks of sneezing from March 20 to May 14. Had many series of allergic testing with no specificity found on scratch tests. During his attacks he had itching of the eyes and nose and typical rhinorrhea associated with nasal obstruction. Tests for the Spring trees were also negative. Despite the negative tests the patient had received several series of desensitization injections with Spring grasses as well as the Spring tree pollens, with no benefit. Nasal examination showed a hyperplastic bilateral ethmoiditis with deflected septum; tonsils had been removed; a septum operation had been performed in February of 1945.	In 1945 he was put on high dosage Vitamin C, 1500 mg. daily with good control of his attacks. The patient stated that he was satisfied with the results. In 1946 the patient came to the office on March 13. He was put on Sodascorbate tablets, 3, 100 mg., 3 times a day. On March 18 he returned to the office and examination showed rhinorrhea, but no sneezing. The dosage was increased to 4 tablets 3 times a day. Nasal examination May 1st, showed no congestion, no rhinorrhea or sneezing. The patient stated that he was having a very good season, even better than the one previously. He had no irritation of the stomach or the bladder, although he had complained of gastric irritation and frequency during the preceding year with the plain ascorbic acid. Patient's comment: "I was never able to sleep from March 21 until May 21, until I started taking the high Vitamin C dosage and later the Sodascorbate tablets."
3.S.R.	35	F	Hay Fever	For last 4 years has had sneezing and running of nose. Hay fever for 17 years. Asthmatic attacks occasionally in hay fever season. Nasal plastic 6 years ago. **Findings:** Nasal mucosa: Hyperplastic and engorged. Turbinates: Inferior turbinates hypertrophied. Discharge: serous discharge; redness and excoriation of both nasal alae. Allergy testing 4+ to dust and feathers and ragweed. Hapamine series given with no benefit.	Started taking 9 Sodascorbate tablets daily June 15, 1946. During the month of August, until Sept. 10 patient was taking 15 tablets per day. During this time the patient stated that she had no sneezing but a feeling of stuffiness of the nose. This is the first season that the patient has not taken hay fever injections. **Patient's Comment:** "I had about 10 bad days but these were completely relieved by Sodascorbate."
4.F.S.	28	F	Hay Fever	Has had hay fever for last 8 years. About two weeks ago had nasal obstruction with stuffiness, sneezing and running. Has had hay fever injection series each season up to the present. **Examination:** Septum: deflected sharply to the left. Turbinates: Inferior turbinates hypertrophied. Mucosa: Hyperplastic, nasal fossae markedly obstructed. Discharge: Mucoid discharge both middle meati, seromucoid discharge. Transillumination: Moderately diminished both sides. Tonsils: removed.	Patient started taking Vitamin C — 100 mg. tablets 2 t. i. d. August 1st, but complained of nausea and gastric disturbance from the tablets. Discontinued taking tablets after 1 week's time and returned to the office 4 weeks later with severe nasal congestion, inferior turbinates congested. Given Sodascorbate tablets 5 t.i.d. Patient then returned 1 week later. During this time the nasal congestion was greatly improved, sneezing very little and patient states that this time is usually the worse part of her season. No gastric disturbance, noticeable from the large doses of Sodascorbate in comparison to the smaller dosage of Vitamin C.

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Case Reports

NAME	Age	Sex	Diagnosis	History & Findings	Treatment & Results
5.W.L.	46	M	Hay Fever	Hay fever for 30 years, sometimes accompanied by asthmatic attacks. **Findings:** Nasal mucosa: Hyperplastic. Septum: markedly deflected to right. Inf. Turbinates: Hypertrophic, profuse sero-mucoid discharge. Bilateral hyperplastic maxillary and ethmoidal sinusitis.	Started Sodascorbate tablets July 15, 1 t.i.d. Patient increased dosage and at the time of the seasonal attack when he would be suffering most, was taking 4 t.i.d. Did not notice any irritation of stomach or bladder. This is the first season that this patient has not had hay fever injections and stated that this is the best season he has had. Very little sneezing. In previous years he would be sneezing continually. Patient regulated the dosage himself in accordance with his needs. **Patient's Comment:** "I found Sodascorbate very, very helpful to my hay fever condition. As a matter of fact that is now the 4th of Sept. and so far I have suffered practically not at all."
6.G.R.	34	M	Asthma Nasal allergy Generally allergic to all intradermal tests	Asthma in childhood, since age of 5. Has coughing spells, some hay fever attacks; is allergic to molds, pollens, etc. Has had some relief with ascorbic acid. **Findings:** Nasal mucosa: Hyperplastic. Nasal discharge: Mucoid discharge both middle meati. Septum: moderately deflected to right	Patient started Sodascorbate tablets in January, taking 3 t.i.d. Since the patient is living out of the state, he reports to us via mail. And as he knows his condition, he is able to judge the number of tablets that gives him the most benefit. He states that taking 1000-1200 mgs. a day he reacts more favorably. With the taking of this amount, he is more efficient, calm and less fatigued than otherwise, and with the taking of the larger doses he has had none of the customary allergic reactions (swollen eyes, headache, etc.) as he had suffered previously. **Patient's Comment:** "I am very grateful to you for the first intelligent approach to a problem which was becoming a very difficult one to me indeed."
7.G.P.	38	F	Hay Fever	Nasal obstruction and discharge, with sneezing and running since November. Has had vaccine injections. Tickling in throat, wheezing in chest. No family history of hay fever or asthma. **Findings:** Nasal mucosa: Hyperplastic, engorged, allergic in type. Septum: Mid position. Turbinates: polypoid changes **Allergic Testing:** 4+ ragweed; 3+ timothy.	Sodascorbate tablets started at first visit, 3 t.i.d. Patient left the city but reported via mail that she was continuing with the Sodascorbate tablets and had increased to 4 t.i.d. with very little sneezing, or rhinorrhea. **Patient's Comment:** "My sneezing seizures seem to be few and far apart."
8.S.D.	48	M	Hay Fever	For last 4 weeks in Sept. has had nasal stuffiness and sneezing moderately. History of hay fever and asthma. **Findings:** Nasal mucosa: Congested. Septum: Deflected to right and left. Turbinates: Inferior turbinates hypertrophied. **Allergy Testing:** Ragweed 4+; timothy 4+. Has had hay fever injections with very little relief at this time of year.	The first of July the patient started Sodascorbate tablets, 3 t.i.d. and continued 4-5 weeks then gradually tapered off, taking 2 t.i.d. then 1 t.i.d. Was well through this period. Then for several days went without Sodascorbate and the nose became congested, breathing difficult and sneezing. The patient then started taking 3 t.i.d. again and all symptoms gradually cleared. No irritation of stomach or bladder noticeable. **Patient's Comment:** "Of all the remedies that have been given me in the past for my allergic condition, I must say that Sodascorbate definitely has proven the most effective."

CONCLUSION

1. The availability of the sodium salt of ascorbic acid for oral use represents a marked advance in the therapeutic approach to allergy.

2. The increased sensitivity to histamine in supra-renalectomized animals suggests a relationship between adrenal cortical hormone-Sodium-Vitamin C and allergy.

3. In refractory cases of allergy and asthma sodium ascorbate was more effective than ascorbic acid.

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A Study of a Group of Poorly Regulated Diabetic Patients

by
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CONSIDERABLE INTELLIGENCE and cooperation on the part of the diabetic patient is necessary for satisfactory control. These faculties were generally absent in the subjects of the present report; as a result an opportunity was provided for the study of relationships between various degrees of hyperglycemia, ketosis and abnormal body weight.

METHODS AND MATERIAL

The subjects were members of the diabetic clinic in the out-patient department of the University Hospital. Almost every patient who attended the clinic during the period of the study was examined — there was no selection of subjects. The group included 16 white patients, 3 male and 13 female, and 34 negroes, 11 male and 23 female.

Calculated diets were not employed in this study. Experience had shown diet lists to be of no practical value. However, the subjects were advised repeatedly to avoid concentrated fat and carbohydrate foodstuffs, and to subsist largely on a diet of lean meat and green vegetables; and attention was repeatedly directed to their condition in relation to ideal weight.

These patients took their insulin (protamine-zinc-insulin) in a single dose daily before breakfast; this plan was adopted primarily because of the factor of simplicity.

Blood for analysis was collected in the diabetic clinic at about three hours after breakfast and the daily injection of insulin. It was assumed that a single specimen collected at this time would have more significance, at least for the level of glucose, than a fasting specimen; this assumption is supported by the observations of Sindoni (1).

The methods employed for chemical analysis were: for blood sugar the Nelson-Somogyi (2) colorimetric procedure, and for acetone bodies the method of Greenberg and Lester (3).

DISCUSSION OF RESULTS

All of the results are assembled in Figure I. It will be observed that about three-fifths of the patients had a level of blood sugar above 200 mg.% and about one-fourth above 300 mg.%. This poor state of control was accounted for quite frequently by

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Electrocardiogram after intravenous administration of glycerol-sodium ascorbate as ocular and intracranial pressure-reducing

By R. Palombi and M. G. Bucci

The prompt and marked pressure-reducing action exerted on the eyeball by glycerol given orally at 50% led Virno, Bucci, Pecori-Giraldi and Cantore to try intravenous administration.

Said authors were prompted to do this by having found that the action of the drug was to be attributed essentially to the presence of the glycerol in the bloodsteam and hence to the increased osmotic power of the blood, and also by the need for a pressure-reducing drug of osmotic action which would permit to attain rapidly and at low doses a marked pressure reduction of the eyeball.

Practically, the problem was to find a way to cause a rapid and intensive reduction of the intraocular tension in subjects where oral administration was impossible or not advisable (pressure reduction in subjects in narcosis, disposition for nausea and vomiting).

In previous investigations of our school it has been demonstrated that urea as well as mannitol, although they certainly are efficacious as pressure-reducing drugs of osmotic action suitable for intravenous administration, are without hazard and phenomena of intolerance owing to the toxicity of the substance, on the one hand, as far as urea is concerned, and on the other (mannitol), the necessity of giving an excessive amount of solution.

Therefore, in view of the brilliant results obtained before with the oral administration of glycerol, the possibility of applying said drug intravenously was considered.

After a series of experimental and clinical studies, the causes responsible for the hematuria brought about by the release of glycerol at different concentrations into the bloodsteam were identified.

It was found, in fact, that it was due to a transitory functional alteration of the renal arterioles which, under the action of a massive dosage of

glycerol, changed from initial conditions of vasospasm to a prolonged stage of vasodilatation which permitted the passage of blood from the glomerular filter, by rhexis or by diapedesis.

It was later possible to observe that to eliminate this hematuria it was necessary to combine with the glycerol a suitable quantity of vitamin C in the form of sodium salt - sodium ascorbate.

This combination was the more fortunate as the ascorbate by itself already possesses a considerable osmotic power which permits marked reductions of the intraocular tension both in normal and in glaucomatose subjects.

In short, it was found that the intravenous administration of Glycerol-Sodium ascorbate represents the chosen osmotic agent for intravenous administration, as it permits obtaining a rapid and marked reduction of the ocular tension with much lower dosages of solution than other osmotic drugs and after very short infusion times.

This can be seen from Table 1, which shows the comparative results with the intravenous administration of the various ocular pressure reducing substances in use in recent years and suitable for intravenous administration.

In a previous study (Bucci and Palombi) we had observed that the intravenous administration of therapeutically active doses of mannitol led in some cases to modifications of the electrocardiogram, sometimes accompanied by pain in the precordium and arm and by tachycardia.

These changes in the EKG consisted mainly in increased frequency evident when checked 30 minutes after the start of infusion, and in the appearance of extra-systoles.

Both disturbances disappeared in the graphs taken at different times from the start of the experiment.

Graph 1. Ocular pressure reducing action exerted by the combination glycerol + sodium ascorbate given intravenously in a patient with acute closed-angle glaucoma in the left eye. The solution was administered at an infusion rate of 90 drops per minute.

Table 1. It is evident from the table that a much smaller amount of solution than for other substances (140 cc for an average weight of 70 kg) is sufficient to obtain a marked and prompt reduction of the intraocular tension in a shorter time (see Graph 1).

Substance	Dose/kg	Quant. solution	Infusion time
Urea (30%)	1 g	230 cc	57 min
Mannitol (20%)	2 g	700 cc	175 min
Sodium ascorbate (20%)	1 g	350 cc	87 min
Glycerol + sodium ascorbate (44%)	0.6 + 0.28 g	140 cc	35 min

One of the causes to which to ascribe these changes of the EKG was seen to be a greater energy requirement of the heart due to an increase of blood volume.

This increase seems to be due to the recall of liquids from the tissues into the bloodsteam by the osmotic action of the drug.

To a greater energy requirement of the hart must be attributed also the appearance of extrasystoles, both atrial but especially ventricular.

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In view of the results obtained by us in the above mentioned research, we thought it of interest to subject to electrocardiographic study persons undergoing treatment with glycerol + sodium ascorbate given intravenously.

The interest is justified, of course, if we consider the notable osmotic power possessed by the combination of these two drugs and the equally notable recall of liquids which must follow their intravenous administration.

Personal Research

We have examined in all 16 subjects, aged between 22 and 77 years, of both sexes.

In the majority of the cases there was no noteworthy electrocardiographic alteration in checks made before the experiment.

Each subject, fasting for at least 6 hours, was given by intravenous

fusion at an average rate of 80-90 drops per minute, a solution of glycerol + sodium ascorbate obtained as follows: 30 cc of highest purity glycerol were brought to 100 cc by addition of sodium ascorbate 20%, at pH 7.4. The total concentration in grams of the combination of the two substances came to 44%.

All subjects were given a dose of 2 cc of solution per kg of body weight, corresponding to 0.6 g/kg glycerol and 0.28 g/kg sodium ascorbate intravenous.

The electrocardiographic examinations were made before the start of the infusion and 30 and 45 minutes, respectively, after the start thereof.

At the same time, tonometric checks were made with the Schiötz indentation tonometer.

In Table 2 we report the average cardiac frequency of each subject obtained from the examination of the EKG tracings in the various derivations and performed at the aforesaid intervals of time from the start of the infusion.

Table 2.

Name	Years	Sex	Diagnosis	Basic cardiac freq./min	After 30 min	After 45 min
C.L.	22	F	Recent gl.	66	56	56
N.A.	55	M	Sec. gl.	70	unchanged	65
C.C.	28	F	Chron. gl	95	85	85
S.F.	34	M	Sec. gl.	92	70	70
Q.A.	51	M	Hemor. gl.	66	54	56
V.M.	77	F	Compl. cat.	82	67	67
G.C.	23	F	Sec. gl.	72	63	63
T.E.	63	M	Chron. gl.	87	76	74
B.L.	29	F	Tr. cat.	78	58	60
P.R.	30	M	Ret. hemor.	70	65	65
D.V.	72	M	Cong. gl.	82	74	74
R.A.	61	F.	Sen. cat.	73	67	65
V.A.	67	F	Sec. gl.	80	50	52
G.L.	48	M	Chr. gl.	70	60	62

I.P.	58	F	Sen. cat.	68	55	58
D.M.	38	M	Sec. gl.	80	68	79

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Results

From an examination of the electrocardiograms carried out by us at the various intervals of time mentioned above, it is evident above all that the administration of the solution glycerol + sodium ascorbate at the aforesaid concentration and at the average rate of infusion of 80-90 drops per minute caused in all subjects examined by us a sometimes marked reduction of the cardiar frequency, to the point of sometimes reaching a bradycardiac rhythm.

This bradycardia appeared already in the check made 15 minutes after the start of the infusion and remained nearly constant in the subsequent check after 45 min.

It has not been possible in the various graphs to evidence alterations of any kind attributable to the action of the substances given.

Only in some rare cases we found an increase of the T wave in some derivations.

Considerations

The only factor which we think we should call attention to is the modification of the cardiac rhythm in the subjects treated with solution of glycerol + sodium ascorbate at the doses and in the manner set forth above.

(Illustration on p. 46)

B.L. 29 years - Base frequency 78/min

After 30 min Mean frequency 58/min

V.A. 67 years - Base frequency 80/min

After 30 min Mean frequency 50/min

V.M. 77 years - Base Frequency 82/min

After 30 min Mean frequency 67/min

In this connection we wish to call to mind the results obtained by us in a previous study of a clinical nature which showed that after oral administra-

tion of glycerol and intravenous administration of mannitol the electrocardiograph showed an increase in cardiac frequency from the start (30 min) of the infusion followed by a return to the base values with some indication of a less frequent cardiac rhythm.

It follows from this that glycerol by itself cannot in our case be the element responsible for the high bradycardia, and ascorbic acid even less.

Thus only the sodium is left which, introduced in the bloodsteam in the form of salt (sodium ascorbate) since it is combined with the ascorbic acid, can affect the normal ion exchanges of the tissues after it is broken down into sodium ion Na^+.

It, as well as other ions (Ca^+, Mg^+, K^+), has the function, in fact among others, to regulate the acid-base equilibrium of the organic fluids and of the tissues.

Na^+ prevalently constitutes the extracellular cation, while K^+ dwells in large part in the intravellular area.

There is thus caused an equilibrium intended, among other things, to regulate the potentials of action of the muscles and nerves.

It is therefore natural that the normal muscular as well as the nervous function can go into effect only when these two cations are in equilibrium.

This electrolytic equilibrium is made possible by the particular properties of the cell membrane, which under normal conditions does not permit other ions which might disturb the state of equilibrium to enter the cell.

Under conditions of altered equilibrium a membrane ion exchange takes place in the sense of Na^+ entering the cell and K^+ coming out.

In our case, therefore, it is possible that the Na which we have introduced into the bloodsteam in the form of sodium ascorbate is sufficient to create the conditions for causing a release into the tissue and hence into the bloodsteam of an abnormal amount of K^+.

Our hypothesis is assisted by the results previously obtained by Cagli, Cantore, Pecori-Giraldi and Virno, who found a constant increase of potassuria in the subjects previously treated with solutions of sodium ascorbate at doses

of 1 g/kg of body weight.

In that case, therefore, knowing the parasympathicomimetic action of potassium on the cardio-circulatory conduction system and the consequent negative chronotropic effect by stimulation of the carotid sinus, it seems to us that the bradycardia observed by us in the present research is justified.

At this point it may seem strange that mannitol, although its osmotic power is notably less than that of the combination glycerol + sodium ascorbate, causes a tachycardia which we believed to be secondary to a greater energy requirement of the heart burdening the circulatory system, while in our case (glycerol + sodium ascorbate) the heart responds with a slowing of its rhythm.

All this is clear when one considers that in the case of mannitol we must add to the amount of liquids recalled from the tissues the huge quantity of solution (700 cc for an individual of 70 kg) introduced into the bloodstream, which is not the case with the combination glycerol + sodium ascorbate (140 cc).

Moreover, we repeat, there must be added the negative chronotropic action of the K^+ ion on the heart muscle.

Also the increased voltage of the T wave in some tracings reveals the presence of the action of the K^+ ion.

Conclusion

From a purely clinical point of view, the infusion of sodium ascorbate and glycerol at the dose of 2 cc of solution per kg of weight was tolerated perfectly by all subjects treated by us, including some who showed anything but a normal basic electrocardiogram.

Only electrocardiographically it has been possible to evidence the appearance of a bradycardia in the checks carried out 30 and 45 minutes after the start of the infusion.

This bradycardia seems to be secondary to the action of the K^+ ion on the cardiac conduction system (negative chronotropic action).

The presence of an abnormal quantity of Na^+ ion in the bloodstream would seem to be the original element responsible for the electrolytic disequilibrium which establishes itself at the cell membrane.

Summary (see p. 49 of original publication)

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CLINICA OCULISTICA DELL'UNIVERSITÀ DI ROMA
Direttore: Prof. G. B. BIETTI

IL TRACCIATO ELETTROCARDIOGRAFICO DOPO SOMMINISTRAZIONE ENDOVENOSA DI GLICEROLO-ASCORBATO DI SODIO, COME MEZZO IPOTONIZZANTE OCULARE ED INTRACRANICO

R. PALOMBI M. G. BUCCI

La pronta e marcata azione ipotonizzante determinata sul globo oculare dal Glicerolo somministrato per via orale al 50% ha indotto VIRNO, BUCCI, PECORI-GIRALDI e CANTORE a tentare la somministrazione per via endovenosa.

A ciò i suddetti AA. furono indotti dall'aver constatato che l'azione del farmaco era essenzialmente da ricondursi alla presenza del glicerolo stesso nel circolo sanguigno e quindi all'aumentato potere osmotico ematico, ed in secondo luogo dalla necessità di disporre di un farmaco ipotonizzante ad azione osmotica che consentisse di raggiungere rapidamente e con modesti dosaggi una marcata ipotonizzazione del bulbo oculare.

Il problema fu praticamente quello di trovare la possibilità di determinare una rapida ed intensa riduzione dell'oftalmotono in soggetti in cui la via orale fosse impossibile o per lo meno poco consigliabile (ipotonizzazioni in soggetti in narcosi, disposizioni alla nausea ed al vomito).

In precedenti indagini della nostra scuola è stato dimostrato come tanto l'urea che il mannitolo, pur possedendo una indubbia efficacia quali farmaci ipotonizzanti ad azione osmotica somministrabili per via endovenosa, non siano scevri da pericoli e da fenomeni di intolleranza da farsi risalire da una parte alla indubbia natura tossica della sostanza per quanto riguarda l'urea e dall'altra (mannitolo) alla necessità di dover somministrare un eccessivo quantitativo di soluzione.

Pertanto, considerando i brillanti risultati ottenuti in prece-

denza con la somministrazione orale di glicerolo, si è pensato alla possibilità di somministrare detto farmaco per via endovenosa.

Dopo una serie di indagini a carattere sperimentale e clinico, si riuscì ad individuare le cause responsabili dell'ematuria determinata dall'immissione in circolo di glicerolo a varie concentrazioni.

Si constatò infatti che ciò era dovuto ad una alterazione funzionale transitoria delle arteriole renali che, per azione del massivo dosaggio di glicerolo passavano da condizioni iniziali di vasospasmo, ad un più lungo stadio di vasodilatazione che consentiva il passaggio di sangue dal filtro glomerulare, per rexin o per diapedesi.

In seguito fu possibile osservare come per eliminare la suddetta ematuria fosse necessario associare al Glicerolo un opportuno quantitativo di Vitamina C sotto forma di sale sodico: l'ascorbato di sodio.

La suddetta associazione risultò tanto più felice in quanto il solo ascorbato già di per sè possiede un notevole potere osmotico che consente marcate riduzioni dell'oftalmotono sia in soggetti normali che in glaucomatosi.

Infine si poté rilevare come la somministrazione di Glicerolo-Ascorbato di sodio per via endovenosa rappresentasse il mezzo osmotico di elezione per somministrazione endovenosa, consentendo di ottenere una rapida e marcata riduzione del tono oculare con dosaggi di soluzione di gran lunga inferiori ad altri farmaci osmotici e dopo tempi di infusione notevolmente brevi.

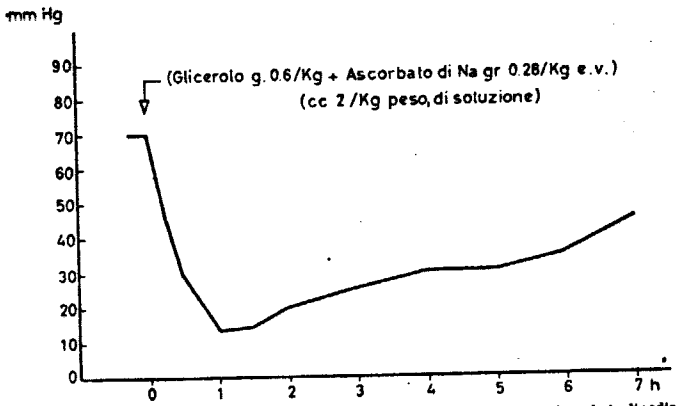
Ciò è possibile rilevare dalla tabella 1 in cui vengono riportati i risultati comparativi sulla somministrazione endovenosa delle varie sostanze ipotonizzanti oculari in uso in questi ultimi anni e somministrabili per via endovenosa.

In una nostra precedente indagine (Bucci e Palombi) avevamo osservato come la somministrazione di dosi terapeuticamente attive di Mannitolo per via endovenosa inducesse in alcuni casi a modificazioni del tracciato elettrocardiografico accompagnate a volte da dolore al precordio, al braccio e da tachicardia.

Le suddette modificazioni del tracciato ECG erano principalmente rappresentate da aumento della frequenza evidente al controllo eseguito a distanza di 30 m' dall'inizio della infusione e dalla comparsa di extrasistoli.

Entrambi i disturbi scomparivano nei tracciati eseguiti a varia distanza di tempo dall'inizio della esperienza.

Una delle cause alle quali far risalire le suddette modificazioni



Graf. 1. - Azione ipotonizzante oculare esercitata dall'associazione Glicerolo + Ascorbato di sodio per via endovena in un paziente affetto all'OS da glaucoma ad angolo chiuso in fase acuta. La soluzione è stata somministrata ad una velocità di infusione di 90 gocce/m'.

Tabella 1. - *Appare evidente dalla tabella come sia sufficiente un quantitativo di soluzione notevolmente inferiore alle altre sostanze (cc. 140 per un peso medio di 70 Kg) per ottenere in un tempo inferiore una marcata e pronta riduzione dell'oftalmotono (vedi Graf. 1).*

Sostanza	Dose/Kg	Quant. soluz.	Tempo inf.
Urea (30%)	gm. 1	cc. 230	m' 57
Mannitolo (20%)	gm. 2	cc. 700	m' 175
Ascorbato di sodio (20%)	gm. 1	cc. 350	m' 87
Glicerolo + Ascorbato di sodio (44%)	gm. 0,6 + gm. 0,28	cc. 140	m' 35

del tracciato ECG fu ricercata in una maggior richiesta energetica del cuore da ricondursi ad un incremento della volemia.

Detto incremento pare essere dovuto al richiamo di liquidi dai tessuti nel circolo ematico per l'azione osmoticamente attiva del farmaco.

Ad una maggior richiesta energetica cardiaca deve essere attribuita anche la comparsa di extrasistoli sia atriali, ma soprattutto ventricolari.

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In forza soprattutto dei risultati da noi ottenuti nella suaccennata indagine, ci è sembrato non privo di interesse sottoporre ad indagine Elettrocardiografica soggetti in corso di trattamento con Glicerolo + Ascorbato di sodio somministrati per via endovenosa.

L'interesse appare naturalmente giustificato se si considera il notevole potere osmotico posseduto dalla associazione dei due suddetti farmaci e dall'altrettanto notevole richiamo di liquidi che deve conseguire alla loro somministrazione endovenosa.

Indagine personale

Abbiamo complessivamente esaminato 16 soggetti di età varia dai 22 ai 77 anni e d'ambo i sessi.

La maggioranza dei casi non presentava alterazioni elettrocardiografiche degne di nota in controlli eseguiti prima dell'esperienza.

A ciascun soggetto, digiuno da almeno 6 ore, veniva somministrata per infusione endovenosa ad una velocità media di 80-90 gocce al m', una soluzione di Glicerolo + Ascorbato di sodio cosí ottenuta: 30 cc di glicerolo purissimo venivano portati a 100 cc mediante l'aggiunta di ascorbato di sodio al 20%, a pH 7,4. La concentrazione in grammi complessiva della associazione delle due sostanze veniva ad essere del 44%.

A tutti i soggetti è stata somministrata una dose di 2 cc. di soluzione/Kg di peso corporeo, corrispondente a 0,6 g/di glicerolo e 0,28 g/Kg di ascorbato di sodio endovena.

I controlli elettrocardiografici venivano eseguiti prima dell'inizio dell'infusione e rispettivamente a distanza di 30'-45' dall'inizio stesso.

Parallelamente venivano eseguiti controlli tonometrici mediante tonometro ad indentazione di Schiötz.

Nella tabella 2 riportiamo la frequenza cardiaca media di ciascun soggetto ricavata dall'esame dei tracciati ECG nelle varie derivazioni ed eseguiti ai suddetti intervalli di tempo dall'inizio dell'infusione.

Tabella 2.

Nome	Anni	Sesso	Diagnosi	Freq. cardiaca di base/m'	Dopo 30'	Dopo 45'
C. L.	22	♀	gl. giov.	66	56	56
N. A.	55	♂	gl. sec.	70	invar.	65
C. C.	28	♀	gl. cron.	95	85	85
S. F.	34	♂	gl. sec.	92	70	70
Q. A.	51	♂	gl. emor.	66	54	56
V. M.	77	♀	cat. compl.	82	67	67
G. C.	23	♀	gl. sec.	72	63	63
T. E.	63	♂	gl. cron.	87	76	74
B. L.	29	♀	cat. tr.	78	58	60
P. R.	30	♂	emor. ret.	70	65	65
D. V.	72	♂	gl. cong.	82	74	74
R. A.	61	♀	cat. sen.	73	67	65
V. A.	67	♀	gl. sec.	80	50	52
G. L.	48	♂	gl. cr.	70	60	62
I. P.	58	♀	cat. sen.	68	55	58
D. M.	38	♂	gl. sec.	80	68	79

Risultati

Dell'esame dei tracciati elettrocardiografici da noi eseguiti ai vari intervalli di tempo suddetti, appare evidente anzitutto come la somministrazione della soluzione di glicerolo + ascorbato di sodio alle concentrazioni suddette ed alla velocità di infusione media di 80-90 gocce al m', abbia determinato in tutti i soggetti da noi esaminati una diminuzione a volte marcata della frequenza cardiaca, fino a giungere a volte ad un ritmo bradicardico.

Detta bradicardia compariva già al controllo eseguito a distanza di 15 m' dall'inizio della infusione e si manteneva pressoché costante al successivo controllo dei 45 m'.

Nei vari tracciati non è stato possibile evidenziare alterazioni di alcun genere imputabili alla azione delle sostanze somministrate.

Solo in qualche raro caso si è assistito ad un incremento dell'onda T in alcune derivazioni.

Considerazioni

L'unico elemento sul quale riteniamo di dover richiamare l'attenzione è rappresentato dalle modificazioni del ritmo cardiaco nei soggetti trattati con soluzione di glicerolo + ascorbato di sodio secondo i dosaggi e le modalità suesposte.

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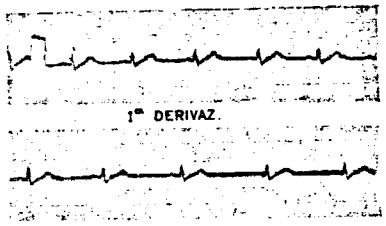
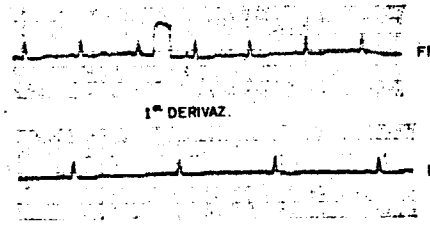


B. L. a. 29



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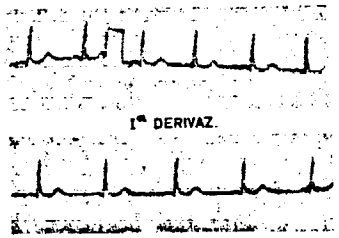

V. M. a. 77

A questo proposito riteniamo opportuno ricordare i risultati da noi ottenuti in una nostra precedente ricerca a carattere clinico in cui fu possibile constatare come dopo la somministrazione orale di glicerolo ed endovenosa di mannitolo, il tracciato elettrocardiografico mostrasse un aumento della frequenza cardiaca fin dall'inizio (30 m') dell'infusione seguito da un ritorno ai valori di base con qualche accenno ad un ritmo cardiaco meno frequente.

Da ciò si può dedurre che il glicerolo di per sè non può essere

nel nostro caso l'elemento responsabile della rilevata bradicardia, e tanto meno l'acido ascorbico.

Non rimane quindi che rivolgersi al sodio che, introdotto in circolo sotto forma di sale (ascorbato di sodio) in quanto legato all'acido ascorbico, dopo la scissione in ione sodico Na^+, può interferire nei normali scambi ionici dei tessuti.

Ad esso, come ad altri ioni (Ca^+, Mg^+, K^+) spetta infatti fra l'altro il compito di regolare l'equilibrio acido-base dei liquidi organici e dei tessuti.

Il Na^+ costituisce in prevalenza il catione extracellulare, mentre il K^+ alberga in gran parte nel distretto intracellulare.

Viene così a determinarsi un equilibrio destinato fra l'altro alla regolazione dei potenziali d'azione dei muscoli e dei nervi.

È quindi naturale che la normale funzione sia muscolare che nervosa possa attuarsi solo in condizioni di equilibrio fra i due suddetti cationi.

Detto equilibrio elettrolitico è reso possibile dalle particolari proprietà della membrana cellulare che non consente, in condizioni di norma, l'ingresso all'interno della cellula di altri ioni che possano turbare lo stato d'equilibrio stesso.

In condizioni di alterato equilibrio, si assiste ad uno scambio ionico di membrana nel senso di un ingresso all'interno della cellula di Na^+ e di una fuoriuscita di K^+.

Nel nostro caso è quindi possibile che il Na da noi introdotto in circolo sotto forma di ascorbato di sodio sia sufficiente a creare le condizioni per determinare una fuoriuscita nel distretto tissurale e quindi nel circolo, di un abnorme quantitativo di K^+.

Questa nostra ipotesi è suffragata dai risultati in precedenza ottenuti da Cagli, Cantore, Pecori-Giraldi e Virno, i quali rilevarono un aumento costante della potassiuria nei soggetti trattati in precedenza con soluzioni di ascorbato di sodio alle dosi di gr. 1/Kg di peso corporeo.

In tal caso, quindi, conoscendo l'azione parasimpaticomimetica esercitata dal potassio nei confronti del sistema di conduzione cardio-circolatorio ed il conseguente effetto cronotropo negativo per stimolazione del seno carotideo, ci pare che la bradicardia da noi rilevata nella presente indagine sia giustificata.

A questo punto può apparire strano come il mannitolo, pur possedendo un potere osmotico notevolmente inferiore rispetto all'associazione glicerolo + ascorbato di sodio, determini una ta-

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chicardia che noi abbiamo ritenuto secondaria ad una maggior richiesta energetica da parte del cuore per in sovraccarico circolatorio, mentre nel nostro caso (glicerolo + ascorbato di sodio) il cuore risponde con un rallentamento del suo ritmo.

Il tutto appare chiaro se si considera come nel caso del mannitolo al quantitativo di liquidi richiamato dai tessuti deve aggiungersi l'ingente quantità di soluzione (cc. 700 per un individuo di 70 Kg) introdotta in circolo; il ché non avviene con l'associazione glicerolo-ascorbato di sodio (cc. 140).

Oltre a ciò è da aggiungersi, ripetiamo, l'azione cronotropa negativa esercitata dallo ione K^+ sul muscolo cardiaco.

Anche l'aumentato voltaggio dell'onda T rilevato in alcuni tracciati, rivela la presenza dell'azione dello ione K^+.

Conclusione

Da un punto di vista puramente clinico, l'infusione di ascorbato di sodio e glicerolo al dosaggio di cc 2 di soluzione per Kg di peso è stata perfettamente tollerata da parte di tutti i soggetti da noi trattati anche da alcuni che presentavano un tracciato elettrocardiografico di base non del tutto normale.

Solo da un punto di vista elettrocardiografico è stato possibile evidenziare l'insorgenza di una bradicardia nei controlli eseguiti a distanza di 30-45 m' dall'inizio della infusione.

Detta bradicardia pare essere secondaria all'azione esercitata dallo ione K^+ sul sistema di conduzione cardiaco (azione cronotropa negativa).

La presenza in circolo di una abnorme quantità di ione Na^+ dovrebbe essere l'elemento primitivo responsabile dello squilibrio elettrolitico che viene a crearsi a livello della membrana cellulare.

RIASSUNTO

La somministrazione per via endovenosa di una soluzione di glicerolo + ascorbato di sodio determina una pronta e marcata riduzione del tono oculare. Il tracciato elettrocardiografico eseguito a distanza di 30-45 m' dall'inizio della infusione della sostanza, ha mostrato in tutti i soggetti trattati una riduzione della frequenza cardiaca che ha raggiunto a volte valori bradicardici.

Il fenomeno può essere ricondotto ad uno squilibrio elettrolitico determinato dalla introduzione in circolo del sodio.

Nessun altro effetto collaterale è stato notato. La sostanza è stata perfettamente tollerata da tutti i soggetti trattati.

RÉSUMÉ

Palombi R. et Bucci M. G. - *Le tracé électrocardiographique après administration intraveineuse de Glycérol + Ascorbate de sodium, comme moyen hypotonisant oculaire et intracranien.*

L'administration par voie intraveineuse d'une solution de glycérol + Ascorbate de sodium provoque une prompte et marquée réduction de la tension oculaire. Le tracé électrocardiographique pratiqué après 30-45 minutes du commencement de l'infusion, a montré chez tous les sujets une réduction de la fréquence cardiaque qui atteindait quelquetois des valeurs bradycardiques.

Le phénomène est probablement du au trouble éléctrolitique produit par l'introduction du sodium dans la circulation sanguigne.

Aucun effet collateral a été observé. La substance a été parfaitement tolérée chez tous les sujets traités.

SUMMARY

Palombi R. and Bucci M. G. - *The ECG after intravenous administration of glycerol + sodium ascorbate, as an ocular and intracranial ipotensive agent.*

Intravenous administration of a glycerol + sodium ascorbate solution produces a marcked reduction of intraocular pressure.

The ECG made after 30-45 m' from the beginning of the infusion schowed in all treated patients a reduction of the cardiac frequency.

The possible cause of this cardiac rhytm's alteration might be an electrolytic involvement produced by the introduction in the blood circle of the sodium.

No other effect has been produced by the intravenous administration of the solution.

ZUSAMMENFASSUNG

Palombi R. und Bucci M. G. - *Das elektrokardiographisches Bild nach der intravenösen Verabreichung von Glyzerol + Natrium Askorbinat, als osmotisches Mittel zur Druckesenkung im Auge und Gehirn.*

Die Verfasser haben festgestellt, dass die intravenöse Verabreichung einer Lösung von Glyzerol + Natrium Askorbinat eine rasche und ausgeprägte Augendrucksenkung verursacht. Die elektrokardiographische Aufnahme, welche man nach 30-45 Minuten vom Anfang der Infusion der Substanz ausübte, zeigte bei allen behandelten Subjekten eine Verminderung der Herzgeschwindigkeit, die manchmal bradykardische Werte erreichte.

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Die erzielten Ergebnisse könnten möglicherweise auf einen elektrolitischen Missverhältnis, welcher von der Einführung des Natriums im Blutkreislauf verursacht ist, zurückzugeführt werden.

Man bemerkte keine anderen Nebeneffekte. Alle behandelten Subjekte vertrugen die Lösung von Glyzerol + Natrium Askorbinat sehr gut.

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CLINICA OCULISTICA DELL'UNIVERSITÀ DI ROMA

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LA SOMMINISTRAZIONE LOCALE DEL PROPANOLOLO NELLA TERAPIA DEL GLAUCOMA

M. G. BUCCI
J. PECORI GIRALDI
A. MISSIROLI
M. VIRNO

Si deve a van EULER (8) l'identificazione del ruolo primario esplicato dalla noradrenalina quale trasmettitore dell'impulso nervoso alla terminazioni simpatiche adrenergiche. Le catecolamine piú importanti implicate nell'attività nervosa simpatica sono la noradrenalina, la adrenalina, la dopamina e in misura minore la isoprenalina. L'attività nervosa nei nervi adrenergici è associata con la liberazione negli spazi sinaptici di noradrenalina che, successivamente, reagisce con i recettori posti sia sulla superficie della cellula effettrice sia entro il citoplasma cellulare. I recettori possono essere sia sistemi enzimatici sia gruppi molecolari specializzati che iniziano la risposta nei tessuti.

ALQUIST (1) ha classificato questi recettori in due gruppi principali a seconda della relativa sensibilità del tessuto alle catecolamine e della specificità del blocco da parte di due gruppi di agenti bloccanti-recettori alfa e recettori beta. In genere, i recettori alfa sono addetti ad un processo di attivazione (ad esempio inducono una contrazione cardiaca) ed i recettori beta ad un processo di inibizione (rilasciamento cardiaco).

Per quanto riguarda il blocco di questi recettori, esistono sostanze che bloccano in modo specifico i recettori alfa ed altre i recettori beta, sebbene la specificità con queste sostanze non sia sempre assoluta. Tra gli agenti che bloccano i recettori alfa, senza influire sui recettori beta, troviamo il piperossano e l'ergotamina, mentre tra gli agenti che bloccano i recettori beta si trovano la dicloroisoprenalina, il pronetanolo e il propanololo, che agiscono solo modestamente ed in alcuni tessuti sui recettori alfa.

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THE INFLUENCE OF ORAL ASCORBIC ACID ADMINISTRATION ON THE ADRENAL CORTICAL RESPONSE TO ACTH[1]

By F. R. Skelton and C. Fortier[2]

Abstract

The effect of orally administered ascorbic acid (as sodium ascorbate) on the adrenal cortical response to ACTH was studied in the hypophysectomized male rat. Plasma and adrenal ascorbic acid and adrenal cholesterol levels were determined three and six hours after ACTH injection. The depletion of adrenal ascorbic acid following such treatment was partially inhibited by the administration of sodium ascorbate while the decrease in cholesterol was unaffected. These results suggest that the uptake of ascorbic acid from the increased quantity available in the plasma was sufficient to partially overcome the depleting effect of ACTH.

Introduction

Recently Dugal and Thérien (2) have reported that the adrenal hypertrophy normally produced by chronic exposure to cold was completely prevented by the administration of ascorbic acid. Further evidence of the conditioning of adrenal cortical function has been presented by Constantinides, Fortier, and Skelton (1) who showed that glucose administration significantly potentiated the adrenal cholesterol depletion produced by ACTH in the hypophysectomized rat. These observations as well as the similarity between the chemical structure of glucose and ascorbic acid have suggested a study of the effect of this vitamin on the adrenal cortical response to ACTH.

Materials and Methods

For this purpose 56 male, piebald rats averaging 150 gm. in weight were hypophysectomized and equally divided into seven groups which were then treated in the following manner. Group I was untreated and acted as the normal control. Groups II and III received a single subcutaneous injection of 2.0 mgm. of Armour's ACTH (Lot No. 60-61) in 0.2 ml. of 0.9% sodium chloride. Groups IV and V were similarly injected with saline (sham ACTH injection) and in addition received ascorbic acid (as sodium ascorbate) by stomach tube two hours later in a dose of 200 mgm. per 100 gm. of body weight. Groups VI and VII received both the ACTH and ascorbic acid treatment. One group of each pair was killed at three hours and the other at six hours postinjection so that all events were studied as a function of time. The time schedule was arranged in such a way that all animals were killed

[1] *Manuscript received February 1, 1951.*

Contribution from l'Institut de Médécine et de Chirurgie Expérimentales, Université de Montréal, Montréal, Qué.

[2] *This work was done during the tenure of an American Heart Association Research Fellowship.*

24 hr. after hypophysectomy and the beginning of fasting. Both adrenals were removed and blood samples collected immediately at the time of killing. Adrenal cholesterol was determined by the method of Sperry (7) and the adrenal and plasma ascorbic acid by the method of Roe and Kuether (6).

Results

The results are summarized in Table I and graphically illustrated in Fig. 1.

TABLE I

EFFECT OF ASCORBIC ACID ON ADRENAL CORTICAL RESPONSE TO ACTH

Treatment	Chemical determinations								
	Plasma ascorbic acid, γ/100 ml.			Adrenal ascorbic acid, mgm. /100 gm.			Adrenal cholesterol, mgm. /100 mgm.		
	Time intervals*								
	0	3	6	0	3	6	0	3	6
ACTH**	460 ±98	617 ±79	550 ±104	384 ±13	217 ±9	312 ±6	5.11 ±0.81	3.60 ±0.82	3.78 ±0.18
Ascorbic acid***	460 ±98	1925 ±680	— —	384 ±13	425 ±31	— —	5.11 ±0.81	4.65 ±1.51	— —
ACTH and ascorbic acid	460 ±98	1400 ±248	4000 ±1680	384 ±13	310 ±20	365 ±35	5.11 ±0.81	3.51 ±0.49	3.27 ±0.74

* *Time is expressed in hours after ACTH or sham ACTH administration.*
** *ACTH (Armour, lot No. 60-61), 2 mgm. per 100 gm. of body weight.*
*** *Ascorbic acid (as sodium ascorbate) 200 mgm. per 100 gm. of body weight.*

Plasma Ascorbic Acid

As previously observed (3, 4) ACTH alone caused a slight but transient increase in the plasma ascorbic acid concentration but did not alter the marked elevation resulting from sodium ascorbate administration.

Adrenal Ascorbic Acid

Sodium ascorbate was also observed to increase slightly above normal, the adrenal content of ascorbic acid. When ACTH and sodium ascorbate were both given, the maximum depletion of adrenal ascorbic acid (19%) was significantly smaller than that observed after ACTH treatment alone (44%).

Adrenal Cholesterol

The adrenal cholesterol content was unaffected by sodium ascorbate administration while the depletion following treatment with ACTH or ACTH plus sodium ascorbate was identical.

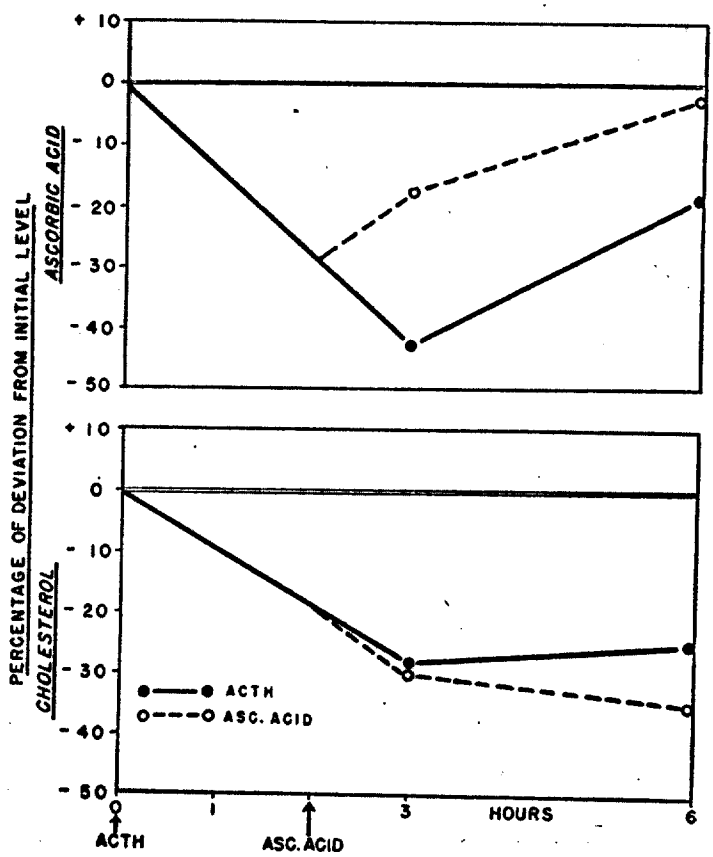

FIG. 1. Plotted in the ordinate are the mean values expressed as % deviation from the hypophysectomized control level. The abscissa indicates time intervals in hours from the moment of ACTH injection.

Conclusions

Elevation of the plasma ascorbic acid level, in the hypophysectomized rat, did not alter the adrenal cholesterol response to ACTH yet partially prevented the adrenal ascorbic acid depletion. This effect is not considered a true conditioning of the adrenal cortical response to ACTH but rather a masking of the normal ascorbic acid decrease by an increased adrenal uptake from the greater quantity available in the plasma. This should be related to Long's observation that in an animal such as the guinea pig which depends on an exogenous source of vitamin C, the ACTH-induced adrenal ascorbic acid depletion is more prolonged than in the rat, which synthesizes its own requirements (5). Both the degree of adrenal ascorbic acid depletion by ACTH and the rate of its repletion would seem to depend on its availability in the plasma.

Summary

The effect of an elevated plasma ascorbic acid level on the adrenal cortical response to ACTH was studied in the hypophysectomized rat. The adrenal cholesterol response to ACTH was unaffected while the ascorbic acid depletion was partially inhibited. These results suggest that the increased adrenal uptake of ascorbic acid from the plasma was sufficient to partially overcome the depleting effects of ACTH.

Acknowledgments

This work was subsidized by a grant from the National Research Council of Canada.

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Vitamins (Japan), Vol. 34, No. 3, pp. 293-297, (1966).

The Metabolism of Ascorbic Acid Esters.

(I) Growth, Excretion and Accumulation After the Administration of Ascorbic Acid Fatty Acid Esters.

By Choten Inagaki and Kanako Kawaguchi, Department of Food and Nutrition, School of Home Economics, Ochanomizu University, Tokyo.

(For a brief English synopsis, refer to the original text on p. 293.)

Many of the biochemical studies on C which have been published in the past relate to AsA*L (L-ascorbic acid) and the numer of studies on L-ascorbic acid fatty acid esters is comparatively small. Only Fitshugh et al. (1) reported on the toxicity of L-ascorbic acid and monopalmitate (As-A and Mono-Pal*2). As a result of the observation on albinos (white mice) after administering feed containing 0.25 and five per cent of mono-palmitate, they reported that, even though there was observed a delay in their growth when five per cent of the same was administered but that the feeding of 2.5 per cent of the same for a period of two years had no observable effect upon their growth.

As regards its physiological effect, further, it is reported that the effects of mono-palmitate and mono-stearate (Mono-ste*3) are not inferior, if not superior, to that of L ascorbic acid (2). This is believed to be due to the fact that the esters contained in the body are hydrolyzed, thereby producing free L-ascorbic acid. According to Fujihira, et al. (3), fatty acid monoesters are hydrolyzed by pancreatic lipase. In addition, Misaka et al. (4) and Riemenschneider et al. (5) reported that the said esters are effective as an antioxidant of fats and are excellent antioxidants.

The current experiment was conducted by the authors for the purpose of clarifying the physiological effect and the mechanism of the metabolism of the monoesters and diesters of L-ascorbic acid.

Physiological Effects of Ascorbic Acid Monopalmitate and Dipalmitate

(1) Experimental Method

Groups of guinea pigs, each consisting of six guinea pigs, both male and female, whose body weight was in the neighborhood of 200 grams, were used and the test period was set at 26 days. Basic feed used consisted of 25.0 per cent of oat, 45.0 per cent of bran, 21.5 per cent of exoleated powdered milk, 7.5 per cent of "sa-flower" oil and 1.0 per cent of dry yeast and three drops of liver oil was administered per week. The regular group was given one milligram of L-ascorbic acid per day to this C-deficient basic feed and the ester-added group was given the addition of an equivalent amount of one milligram of L-ascorbic acid for the purpose of examining the rate of their growth.

(2) Experimental Result

As can be observed in Figure 1, no group showed any sign of a drop in the body weight, and all of them showed similar growth curves, without the development of any sign of scurvy.

(Insert Figure 1 on p. 294. Growth curve of guinea pigs.)

Enzyme Decomposition of Ascorbic Acid Mono- and Dipalmitate

Since it was learned that these esters have a physiological effect as in the case of AsA(L-ascorbic acid), an experiment on the decomposition of esters in vitro was carried out by using purified lipase, guinea pig small intestines and pancreatic smashed solutions as the sources of enzyme.

(1) Experimental Method

The method whereby, after having caused an enzyme solution to work on L-ascorbic acid (AsA) esters, the portion of an aqueous solution containing AsA (L-ascorbic acid) was taken out and the amount of L-ascorbic acid (AsA) was determined by the DNP*4 (dinitrophenyl-hydrazine) method as a transparent solution was examined.

1) Basic Solution

Each ester was added to a concentration of 0.05 M to a two per cent solu-

tion of pH 7.0, 1/15 M phosphoric acid buffer solution of PVA*5 (polyvinyl alcohol) and the same was made into an emulsion by using a homogenizer.

2) Enzyme Solution

Thirty (30) milligrams of purified bacterial lapse was dissolved in 10 milliliters of water. As an organ crude enzyme solution, the smashed solution of the small intestines and the pancreas of guinea pigs whose body weight was in the neighborhood of 600 grams was used.

3) Composition of the Reaction Solution

The composition of the reaction solution was as follows:

(Insert the Table on the right-hand column on p. 294. a. Composition (ml). 1. Substrate emulsion. 2. Buffer solution. 3. Purified lipase. 4. Smashed solution of the organs.)

4) Method of Measurement

After leaving it at the temperature of 37 degrees centigrade for a period of one hour, the reaction was terminated at five per cent methaphosphoric acid, 20 milliliters of petroleum ether was added, stirring was carried out, the water layer was centrifugally precipitated at 8,000 r.p.m. for a period of five minutes, the upper limpid solution was used for the measurement (7) of AsA (L-ascorbic acid) by the DNP method.

(2) Experimental Result

The degrees of the decomposition of esters by the lipase and the smashed solution of the organ were compared and shown in Table 1 below:

(Insert Table 1 on p. 294. Hydrolysis of ascorbic acid esters by lipase and tissue homogenates of guinea pigs.)

In the case of lipase, the decomposition degrees are low in mono- and di-palmitate (Di-Pal*4), with almost no decomposition being shown in the case of di-palmitate. In the experiment using the smashed solutions of the organ, there was found a great difference between the mono- and di-palmitate, with

mono-palmitate being satisfactorily decomposed, whereas the degree of the decomposition of di-palmitate was low. In each case, the degree of decomposition became higher when they were caused to act simultaneously with the small intestines and pancreas.

The above experiment shows that, different from AsA (L ascorbic acid), these esters are believed to be metabolized after having received enzyme decomposition. Next, a metabolism experiment was carried out by using guinea pigs.

Metabolism of Ascorbic Acid and its Esters in Guinea Pigs.

(1) Change in the Amount of Ascorbic Acid Excreted in Urine in Passage of Time After the Administration of Ascorbic Acid and Its Esters.

An experiment was carried out on the change in the amount of AsA secreted in the urine of guinea pigs in terms of time passed after administration for the purpose of examining whether or not there was any difference in the rate of metabolism between AsA (L-ascorbic acid) and its esters.

1) Experimental Method

The experimental animals were classified into the AsA (L-ascorbic acid) group, the Mono-pal (mono-palmitate) group and the Di-Pal (dipalmitate) group, each group consisting of six animals. The composition of the feed given was the same as in the previous case. Two milligrams of AsA (L-ascorbic acid) per day was fed for a period of approximately one week. Then, an equivalent amount of 20 milligrams of AsA (L-ascorbic acid) was once fed to each group and the amount of AsA (L-ascorbic acid) which was excreted into the urine was examined in terms of time elapsed.

Further, relative tothe metabolic cage, guidance was obtained from Professor Kandachi of the University of Tokyo and the Teruuchi method (8) which employs DNP was used as the method for the determination of AsA (L-ascorbic acid) contained in urine.

2) Experimental Result

The change in excretion in urine during the period of 144 hours after the administration is shown in Table 2 and Figure 2.

(Insert Table 2 on p. 295. Amounts of ascorbic acid excreted in the urine of guinea pigs after the administration of ascorbic acid and its esters.)

(Insert Figure 2 on p. 295. Urinary excretion of ascorbic acid.)

As can be observed in the Table, there was observed no significant difference amonst the three groups up to a period of 24 hours after the administration of 20 milligrams. In the 48th hour, however, there was observed a sudden drop in the amount of excretion in the AsA (L-ascorbic acid) group, with the degree of the drop being small in the mono- and di-pal (mono-palmitate and di-palmitate) groups. This tendency continued thereafter. After 144 hours, the amount of AsA (L-ascorbic acid) excreted in the AsA (L-ascorbic acid) and mono-pal (monopalmitate) groups was reduced, whereas a value which was higher than those of the other groups was shown in the di-pal (di-palmitate) group alone. This is believed to be indicative of the fact that there was a difference in the rate of metabolism between mono- and di-.

(2) Change in the Amount of Ascorbic Acid in Terms of Time Elapsed in the Organ(s) and Plasma of Guinea Pigs after the Administration of Ascorbic Acid and Its Esters.

1) Experimental Method

The animals were divided into three groups, each consisting of six animals, including the AsA (L-ascorbic acid) group, the mono-pal (mono-plamitate) group and the di-pal (di-palmitate) group. The composition of the feed given was the same as stated previously. After two milligrams of AsA (L-ascorbic acid) per day was administered for a period from 14 to 18 days, an amount equivalent to 20 milligrams of AsA (L-ascorbic acid) and its ester was given. After the administration, the amounts of the AsA (L-ascorbic acid) contained in the

liver, adrenal and plasma 24, 48 and 168 hours afterward were measured.

First, 0.8 milliliters of "nenputal" was injected into the abdomenal cavity of the animal(s), the chest was opened, blood was collected from the heart by using a needle for the hepalin treatment, the organ(s) was taken out and the AsA (L-ascorbic acid) was measured by the DNP method in accordance with the ordinary practice.

2) Experimental Result

The changes in the amount of AsA (L-ascorbic acid) in the liver, adrenal and plasma after the administration of AsA (L-ascorbic acid) and its ester, are shown in Table 3.

As can be seen in the Table, the AsA (L-ascorbic acid) in liver was 4.18 milligram per cent after 24 hours and 2.62 milligram per cent after 48 hours, showing a drop of approximately 40 per cent. In the Di-Pal (di-palmitate) group, however, it was 2.95 milligram per cent after 24 hours and 4.73 milligram per cent after 48 hours, showing an increase of approximately 30 percent. In the mono-pal (mono-palmitate) group, no such sharp drop as in the AsA (L-ascorbic acid) group was observed.

AsA in the adrenal was 36.4 milligram per cent after 24 hours and 29.79 milligram per cent after 48 hours, showing a tendency to drop as in the case of the liver. On the other hand, there was observed an increase from 23.03 milligram per cent after 24 hours to 28.85 milligram per cent after 48 hours in the Di-pal (di-palmitate) group. After the passage of 168 hours, however, there was a drop of approximately the same in all three cases. As for the amount of AsA (L-ascorbic acid) in plasma, there was observed a significant difference between the di-pal (di-palmitate) group and the other two groups in plasma, the significant difference P being smaller than 0.01.

(Insert Table 3 on p. 296. Changes in ascorbic acid in the liver adrenal and plasma of guinea pigs after the administration of ascorbic acid and its esters.)

Observations:

In view of the fact that, regarding the enzyme system which participates in the decomposition of AsA (L ascorbic acid) esters, the best performance is observed in the combination of the crude enzyme solution of small intestines and pancreas, it seems that these esters and the mono-bodies, in particular, are decomposed in the duodenum part which is connected with the pancreas.

The mono-bodies show a bonding at the sixth position of AsA (L-ascorbic acid); however, the di-bodies show a bonding of fatty acids at the second position and the sixth position. Different from mono-pal (mono-palmitate), it is difficult for AsA to be free in enzyme decomposition in vitro in the case of di-pal (di-palmitate).

Judging from the experiment of the growth of guinea pigs, however, di-bodies are utilized like AsA (L-ascorbic acid). It seems, therefore, that the same is decomposed by a separate enzyme system.

In the case where an excessively large amount of AsA (L-ascorbic acid) is given on the animals, it is not clear why the amount of its excretion into the urine is small. According to a report made public by Burn et al. (9), who examined the 14C which is excreted into the respiration and urine after the administration of AsA (L-ascorbic acid)-14C to guinea pigs, more than 30 per cent appeared in the respiration (breath), with only 10 per cent appearing in the urine, during the period of 48 hours. Of this, 30 per cent was AsA (L-ascorbic acid) and the remainder was composed of 20 per cent of diketoglonic acid and nine per cent of oxalic acid.

Regarding the difference in the amount of AsA (L-ascorbic acid) discharged into urine in terms of time elapsed, there was observed a sudden drop in AsA (L-ascorbic acid) after 24 hours, whereas there was hardly any reduction in the case of AsA esters. The same tendency was observed in the case of organ AsA (L-ascorbic acid). In other words, there was observed a sudden increase in each organ 24 hours after the administration in the group where the

animals were fed with AsA (L-ascorbic acid), with a reduction being observed thereafter. In the group where the animals were given the ester, there was no mentionable increase in the neighborhood of 24 hours, with the subsequent increase being marked. However, after 168 hours, the extent of the reduction was almost the same in all three cases. On this basis, it can be understood that these esters show a different metabolism or they are subjected to enzyme decomposition on a gradual basis after they are taken in as they are.

Conclusion

1) The effects of the mono- and di-palmitates of AsA (L-ascorbic acid) are similar to the effect of AsA (L-ascorbic acid).

2) The result of an experiment on the enzyme decomposition of AsA (L-ascorbic acid) decomposition by using lipase and a guinea pig organ smashed solution showed that, in the case of a mixed ground solution of small intestines and pancreas, approximately 80 per cent of mono-palmitate was decomposed but that the decomposition of di-pal (di-palmitate) was in the neighborhood of two per cent.

3) The result of an observation of the changes in the amount of Asa (L-ascorbic acid) contained in urine, liver, adrenal and plasma of ordinary guinea pigs in terms of time elapsed after the administration of AsA (L-ascorbic acid) and an amount equivalent to 20 milligrams of its ester showed that the excretion of AsA in urine is delayed in the ester group, with a significant difference being observed vis-a-vis the AsA group.

In the value of AsA contained in the liver and adrenal, there was a reduction after 48 hours as compared with the value obtained after 24 hours when AsA was administered. In the Di- and mono-palmitate groups, however, there was an increase after 48 hours, contrary to the above case. After 168 hours, however, the extent of the reduction was almost the same in three groups. There was no great difference amongst the three groups in terms of time elapsed in the concentration of AsA (L-ascorbic acid) contained in plasma.

The essence of the above thesis was published before the 18th convention of the Nippon Vitamin Society. The authors would like to express their thanks to Nikko Chemicals Company, Limited, for the donation of di-palmitate crystals.

(The manuscript was received on May 23, 1966.)

Footnotes and References:

*1) AsA: L-ascorbic acid. *2) Mono-pal: mono-palmitate. *3) mono-ste: Mono-stearate. *4) DNP: dinitrophenylhydrazyne. *5) PVA: Polyvinylalcohol. *6) Di-pal: dipalmitate.

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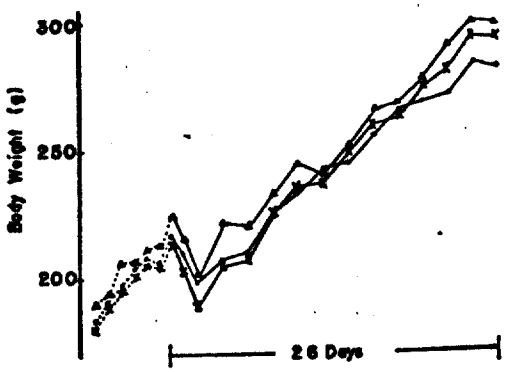
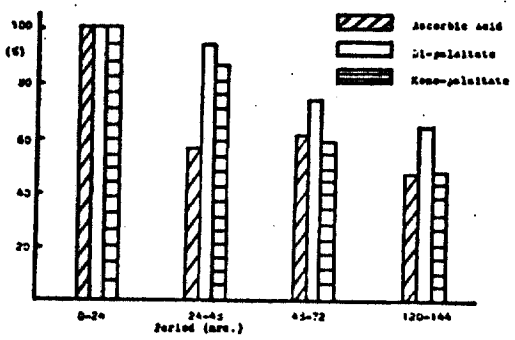
Fig. 1 Growth curve of guinea pigs



● 1mg AsA △ 2.35mg AsA-monopalmitate
× 3.51mg AsA-dipalmitate

Fig. 2 Urinary excretion of ascorbic acid



ascorbyl palm. 22

アスコルビン酸エステルの代謝

(I) アスコルビン酸脂肪酸エステル投与時の成長，排泄および体内貯留

お茶の水女子大学栄養化学教室
稲垣長典・川口加南子

Vitamins (Japan) 34, (3), 293-297 (1966)

STUDIES ON THE METABOLISM OF FATTY ACID ESTERS OF L-ASCORBIC ACID

(1) EXCRETION AND ACCUMULATION OF ASCORBIC ACID AFTER ADMINISTRATION OF MONO- AND DIPALMITATES OF ASCORBIC ACID

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In order to compare the metabolism of L-ascorbic acid with that of its fatty acid esters, the amounts of ascorbic acid in urine, liver, adrenal and blood plasma of guinea pigs were determined after the oral administration of L-ascorbic acid, L-ascorbyl mono- and dipalmitates, equivalent to 20mg of ascorbic acid. In three groups, the urinary ascorbic acid excreted in 0-24 hrs. after administration was higher than that in 24-48 hrs. The changes of ascorbic acid content of the organs showed the similar tendency with that of urine in group of ascorbic acid administration, but in groups of esters administration, the changes showed a somewhat reverse tendency. Enzymatic hydrolysis of ascorbic acid ester was also studied with tissue homogenate of guinea pig. When the mixture of homogenates of small intestine and pancreas was used, the yields of free ascorbic acid were 80% with L-ascorbyl monopalmitate but only 20% with L-ascorbyl dipalmitate.

Cの生化学的研究の多くは主としてAsA*1)にかんするものでAsA-脂肪酸エステルについての研究は少なく，ただFitishughら[1]がAsA-Mono-Pal*2)の毒性について報告し，シロネズミにMono-Palを飼料の0.25および5%レベルで与えて観察した結果，5%にすると成長の遅滞がみられたが0.25%では2年間飼育しても異常はみられなかつたことを明らかにしている．またその生理効果はMono-Pal, Mono-Ste*3)いずれもAsAに比べ優るとも劣らない[2]といわれているがこれは体内でエステルが加水分解され遊離AsAを生ずるためと考えられている．藤平ら[3]によると脂肪酸モノエステルは試験管内で膵リパーゼにより加水分解を受けてAsAを遊離するという．また三坂ら[4]，Riemenschneiderら[5]はこのエステルは脂肪の抗酸化剤として有効で抗酸化性に優れていると報告している．

ここにおいて著者らはAsAのモノエステルとジエステルを用いてそれらの生理効果ならびに代謝機構を解明したいと考えて実験を行なつた．

アスコルビン酸モノパルミテートおよびジパルミテートの生理効果

(1) 実験方法

実験動物とその飼育法

体重200g前後の雌雄モルモットを各群6匹ずつ用い試験期間を26日間とした．基礎飼料は燕麦粉25.0,

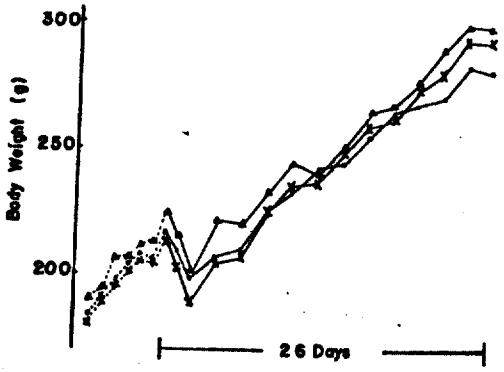
*1) AsA = L-Ascorbic acid　*2) Mono-Pal = Monopalmitate　*3) Mono-Ste = Monostearate

ふすま 45.0，脱脂粉乳21.5，サフラワー油7.5，乾燥酵母 1.0%の配合とし週2回3滴の肝油を与えた．正常群にはこのC欠乏基礎飼料に1日1mgのAsAを与え，エステル添加群ではAsA 1mg相当量を添加し成長試験を行なつた．

(2) 実験結果

図1にみるようにいずれの群も体重の低下するものがなく，ほとんど同等の成長曲線を示し外観上もよく壊血症の発現はみられなかつた．

Fig. 1 Growth curve of guinea pigs



● 1mg AsA　△ 2.35mg AsA-monopalmitate
× 3.51mg AsA-dipalmitate

アスコルビン酸モノ-およびジパルミテートの酵素分解

これらのエステルがAsA同様生理効果があることを知つたのでこれらエステルの酵素分解系を知るために精製リパーゼ，モルモットの小腸および膵磨砕液を酵素源として*in vitro*でエステルの分解実験を行なつた．

(1) 実験方法

AsAエステルに酵素液をを作用させたのち，AsAを含む水溶液部分を取り出し透明な液としてDNP*4)法によりAsAを定量する方法を検討した．

1) 基質溶液

各エステルをPVA*5)のpH 7.0, 1/15*M*リン酸緩衝液2%溶液[6]中 0.05*M*濃度に加えてホモゲナイザーでエマルジョンとする．

2) 酵素液

精製細菌リパーゼ 30mg を水10m*l* にとかした．臓器粗酵素液としては体重600g前後のモルモットの小腸および膵臓を磨砕液として用いた．

3) 反応液組成

反応液はつぎの組成とした．

組成(m*l*)	1	2
基質エマルジョン	5.0	1.0
緩衝液	4.0	1.0
精製リパーゼ	1.0	—
臓器磨砕液	—	2.0

4) 測定法

37℃，1時間温置したのち5%メタリン酸で反応を停止し石油エーテル 20m*l* を加え振とうし水層を 8000 *rpm*，5分遠沈しこの上清についてDNP法によりAsAの測定[7]を行なつた．

(2) 実験結果

リパーゼおよび臓器磨砕液によるエステル分解度を比較した結果を表1に示す．

リパーゼではMono-，Di-Pal*4)いずれも分解度低くとくにDi-Palはほとんど分解されなかつた．臓器磨砕

Table 1 Hydrolysis of ascorbic acid esters by lipase and tissue homogenates of guinea pig

Substrate	Bile acid	Lipase		Homogenate					
				Intestine(A)		Pancreas(B)		A + B	
		AsA (mg)	Hydrolysis (%)	AsA (mg)	Hydrolysis (%)	AsA (mg)	Hydrolysis (%)	AsA (mg)	Hydrolysis (%)
Mono-palmitate	−	1.17	2.66	4.36	49.54	6.71	76.17	7.05	80.03
	+	—	—	—	—	6.60	74.93	—	—
Di-palmitate	−	0.11	0.25	0.03	0.34	0.10	1.10	0.16	1.81
	+	—	—	0.01	0.08	0.04	0.47	0.10	1.09

*4) DNP = Dinitrophenylhydrazine　*5) PVA = Polyvinylalcohol　*7) Di-Pal = Dipalmitate

液を用いた実験ではMono-とDi-でひじように大きな差があり，Mono-Palはよく分解されたが，Di-Palの分解度は低かつた．いずれも小腸と膵臓を同時に作用させると分解度は高くなつた．以上の実験からこれらエステルはAsAと異なり，酵素分解をうけたのち代謝されるものと考え，つぎにモルモットを用いて代謝実験を行なつた．

モルモットにおけるアスコルビン酸およびそのエステルの代謝

(1) アスコルビン酸およびそのエステル投与時の尿中アスコルビン酸排泄量の経時的変化

AsAとそのエステルの間で代謝速度に相違があるかどうかを検討する目的で投与後の尿中AsA排泄量の経時的変化をモルモットを用いて実験した．

1) 実験方法

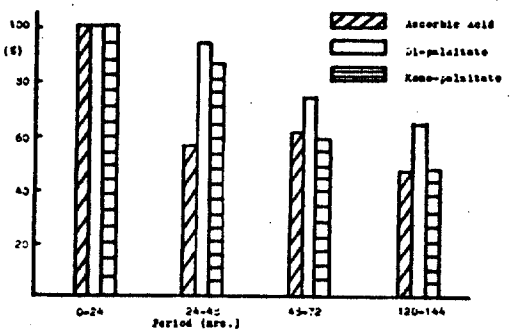
動物は1群を6匹としAsA群，Mono-Pal群，Di-Pal群の3群とした．飼料組成は前述と同様とし，AsAを1日2mg与え約1週間飼育したのち各群にAsA20mg相当量を1回投与し尿中に排泄されるAsA量を経時的に測定した．なお代謝かごについては東大神立教授の指導を得たもので，尿中AsA定量法はDNPを用いる照内法[8]によつた．

2) 実験結果

投与後144時間までの尿中排泄の経時的変化は表2および図2に示すとおりである．

表にみるように20mg投与24時間までは3群間に有意差がなかつた．48時間目になるとAsA群では急激に排泄量が減少したがMono-およびDi-Pal群ではその減少程度が少なかつた．この傾向はその後も続いたが144時間後ではAsA群，Mono-Pal群ともにAsA排泄量は減少したのに反し，Di-Pal群のみはいぜん他の群より

Fig. 2　Urinary excretion of ascorbic acid



高い値を示した．これはMono-とDi-とに代謝速度の相違があることを示すものと思われる．

(2) アスコルビン酸およびそのエステル投与によるモルモットの臓器および血漿中のアスコルビン酸量の経時的変化

モルモットの肝，副腎および血漿中のAsA量の経時的変化を観察した．

1) 実験方法

動物は1群6匹としAsA群，Mono-Pal群，Di-PAl群の3群に分けた．飼料組成は前述と同様で1日2mgのAsAを14～18日間投与したのち，AsAおよびそのエステル20mg相当量を与えた．投与後24，48，168時間後の肝，副腎，血漿のAsA量を測定した．

まず試験動物腹腔内にネンブタール0.8mlを注射し開胸しヘパリン処理注射針で心臓より採血し臓器を取り出しそれぞれ常法に従いDNP法によつてAsAを測定した．

2) 実験結果

AsAおよびそのエステル添加後の肝，副腎，血漿中のAsA量の変化は表3に示す通りである．

表にみるように肝のAsAはAsA群では24時間後4.18mg%，48時間後2.62mg%で約40%も減少したのにたい

Table 2　Amounts of ascorbic acid excreted in urine of guinea pigs after the administration of ascorbic acid and its esters

Period(hrs)	Groups		
	Ascorbic acid (mg)	Di-palmitate (mg)	Mono-palmitate (mg)
0 - 24	0.057±0.012*	0.063±0.008	0.063±0.015
24 - 48	0.032±0.005	0.059±0.007	0.054±0.019
48 - 72	0.035±0.011	0.047±0.007	0.037±0.010
120 - 144	0.027±0.007	0.041±0.024	0.030±0.007

* Standard deviation

Table 3 Changes in ascorbic acid content of liver, adrenal and plasma of guinea pigs after administration of ascorbic acid and its esters

Period (hrs)	Liver (mg%)			Adrenal (mg%)			Plasma (mg%)		
	Ascorbic acid	Mono-ester	Di-ester	Ascorbic acid	Mono-ester	Di-ester	Ascorbic acid	Mono-ester	Di-ester
24	4.18 (0.71)*	4.97 (1.60)	2.95 (1.86)	36.46 (21.94)	35.35 (8.49)	23.03 (6.11)	0.11 (0.03)	0.19 (0.03)	0.0 (0.03
48	2.62 (0.13)	4.46 (2.80)	4.73 (0.18)	29.79 (12.82)	54.59 (22.06)	28.85 (2.35)	0.08 (0.02)	0.13 (0.03)	0.0 (0.04
168	1.08 (0.46)	0.81 (0.24)	1.67 (0.85)	11.74 (3.60)	9.51 (3.25)	17.46 (6.58)	0.08 (0.01)	0.07 (0.01)	0.0 (0.02

* Standard deviation

してDi-Pal群では24時間後2.95mg%，48時間後4.73mg%となり逆に30%近く増加した．Mono-Pal群でもAsA群のような急激な減少はみられなかつた．また副腎のAsAはAsA群では24時間後36.46mg%，48時間後29.79mg%で肝のばあいと同様に減少していたが，Di-Pal群では24時間後23.03mg%，48時間後28.85mg%と増加していた．しかし168時間後には3者ともほとんど同程度に減少した．血漿中のAsA量は24時間後にDi-Pal群と他2群との間に有意差（P <0.01）がみられた．

考　　察

AsAエステルの分解に関与する酵素系については小腸と膵の粗酵素液を合わせたばあいもつともよく働くことからこれらエステルとくにMono-体は膵とつながる十二指腸部で分解されるものと思われる．

Mono-体はAsA 6位の結合であるがDi-体は2位と6位に脂肪酸が結合したものであるから，Di-PalはMono-Palと異なり*in vitro*の酵素分解ではAsAが遊離しにくい．しかしながらDi-体もモルモットの成長実験からみるとAsA同様利用されるからべつの酵素系によつて分解されるようである．

また過剰のAsAを動物に負荷したばあい尿中排泄量が少ないのは疑問であるが，Burnら[9]のAsA-^{14}Cをモルモットに注射したときの呼気と尿中に出る^{14}Cを調査した報告によると48時間中に30%以上が呼気に出て尿中には10%しか出ず，そのうち30%がAsAで，残り20%がジケトグロン酸，9%が修酸であつたということからある程度説明される．

尿中AsA排泄量の時間的差異はAsAは24時間以後に急激に減少したがAsAエステルではほとんど減少しなかつた．この傾向は臓器AsAについてもいえる．すなわちAsA投与群は24時間以後急激に各臓器に増加しその後は減少したがエステル投与群は逆に24時間後はあまり増加せず，その後増加がいちじるしかつたしかし168時間後には3者ともほとんど同程度まで少した．この事実からみてこれらのエステルがAsA異なる代謝すなわちそのまま取りこまれたのちに徐に酵素分解を受けるものと考えられる．

結　　論

1) AsAのMono-およびDi-palmitateはモルモッにたいしAsAと同等の効果があることを認めた．

2) AsAエステルの酵素分解をリパーゼおよびモモット臓器磨砕液について実験した結果，小腸・膵混合磨砕液ではMono-Palは約80%分解されたが，DPalの分解は2%程度であつた．

3) AsAおよびそのエステル20mg相当量を投与し正常モルモットの尿中，肝，副腎および血漿中のAs量を測定し経時的変化をみた結果，尿中AsA排泄はステル群において遅延し，AsA群との間に有意差がめられた．肝および副腎のAsA値はAsA投与のばあ48時間後には24時間後よりも減少していたがDi-およびMono-Pal群においては逆に48時間後に増加してた．しかし168時間後には3群ともほとんど同程度減少した．血漿中AsA濃度の経時的に3群とも大き差はなかつた．

本論文の要旨は第18回日本ビタミン学会において表した．Di-Pal結晶は日光ケミカルズKKより分譲れたものでここに感謝する．

（昭41. 5. 23. 受理）

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モルモットにおけるパントテン酸欠乏と妊娠

妊娠ネズミをPaA欠乏食で養うと産児にいろいろな先天性欠陥を発生するが欠乏食を与える時期および期間によつて異なる（M. M. Nelson, H. V. Wright, C. D. C. Baird, H. M. Evans : J. Nutr. **62**, 395, 1957). L. S. Hurley, H. E. Volkert, J. T. Eichner (J. Nutr. **86**, 201, 1965）は妊娠モルモットにたいするPaA欠乏の影響を研究した．これよりさきに Hurley, Volkert (Biochim. Biophys. Acta **104**, 372, 1965）はモルモット胎児肝のPaAおよびCoA含量は妊娠33～58日のあいだはほとんど変化がないが58日目に突然増加することを認め出産直前にPaAの臨界点があることを認めている．まず成熟した雌雄のモルモットにPaA欠乏飼料を与えたところ雄および非妊娠動物の生存期間は24～25日であつたのにたいして妊娠動物ではわずか13日しか生存しなかつた．ほとんどすべての動物に副腎出血を認めまた約1/4に消化管の出血があつた．肝中のCoAおよびPaAは約半減した．肝脂質はPaA欠乏動物に多かつたが対飼育動物と同等であつたから飢餓の影響と思われる．

つぎの実験では妊娠モルモットを 6, 7, 9 または 10 週目の1週間PaA欠乏食で養つたところ6, 7週目のばあいは出産に影響を与えなかつたが9週目に欠乏食を与えたものでは6匹中産児をえたのは1匹だけで1匹は死亡し2匹は流産し2匹は死産であつた．生まれた3匹のうち1匹は神経症状を呈し6日後に死亡した．10週目に欠乏食を与えたばあいはかえつて障害がすくなく4匹すべて出産した．肝PaA含量は対照動物では出生時は成熟動物の60%程度であつたが7日間で33%の増加を示した．欠乏食を与えた動物でも出生時の肝PaAは低く7日後に増加したが対照と同等に達したのは6および7週目に欠乏食を与えた群だけであつた．

PaA欠乏のもつとも著明な影響の認められたのは妊娠9週目に与えたものでこの時期に胎児がPaAことにCoAをもつとも多量に要求するものと思われる．ほかのB群ビタミンの要求が妊娠初期に著明である（H. Kalter, J. Warkany : Phys. Rev. **29**, 69, 1959)のに反しPaAの最大要求が妊娠末期にある点に興味がある．

幼モルモットのPaA必需量は飼料にたいし 1.5～2.0%で成熟動物におけるよりも低い（M. E. Reid, G. M. Briggs : J. Nutr. **52**, 507, 1954）がシロネズミに比すると2倍以上である．ネズミでは1ヵ月のPaA欠乏食投与は妊娠に影響しない（Everson, *et al.* : J. Nutr. **53**, 341, 1954）というのにたいしてモルモットではPaA欠乏の影響を受けやすいのはその必需量が大きいためと思われる．したがつてモルモットにおけるPaAおよびCoA代謝の研究が望まれる．　(Nutr. Rev. **24**, 169, 1966)　（高田）

エンドウ中に Oxalyl-CoA synthetase の証明

エンドウ種実中に蓚酸＋ATP＋CoA ⇄ Oxalyl-CoA＋AMP＋iPPの反応を営む新しい酵素を証明しOxalate : CoA ligase と命名した．この酵素はルーピン種実，カボチャ，コムギ胚芽中にも存在することを知つた．Oxalyl-CoAはFormyl-CoAとなりCO_2を遊離しFormyl-CoAはギ酸となり結局蓚酸→ギ酸の反応を行なうものであるが植物中でこのほかいかなる反応に関与しているかはまだわかつていない．(Gionavelli, J. : Biochim. Biophys. Acta **118**, 124, 1966)　（高田）

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TRANSFER OF ASCORBIC ACID AND RELATED COMPOUNDS ACROSS THE BLOOD-AQUEOUS BARRIER*

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The high concentration of ascorbic acid in the aqueous humor compared with that in the blood has been offered as presumptive evidence that this compound enters the aqueous humor as a result of a secretory process. Previous studies have shown that while this is true for adult animals the concentration of ascorbic acid in the aqueous humor in young animals is essentially the same as in the blood.[1] The difference in young animals has been explained by assuming that the blood-aqueous barrier is sufficiently permeable to permit rapid diffusion from the aqueous back into the blood.[2]

The implications inherent in the belief that the total transfer of ascorbic acid across the blood-aqueous barrier occurs as a result of a secretory process in adult animals, on the one hand, whereas, a predominant portion of the transfer occurs as a result of diffusion in young animals, on the other hand, have not been considered previously. Thus, if the transfer took place by secretion, it would be expected that the amount of ascorbic acid which could be transferred across the blood-aqueous barrier would be limited by some factor other than the quantity present in the blood; that is, that the secretory process is capable under ordinary conditions of handling but so much ascorbic acid. If the transfer occurs as a result of diffusion, it would be expected that there would be a direct relationship between the concentration in the aqueous and the blood irrespective of the concentration in the latter fluid. One of the objects of the present paper was to subject these inferences to experimental verification.

A second objective concerns the question of the specificity of the secretory process for compounds structurally like ascorbic acid. It is well known that other carbohydrates having similar size and solubility (namely, glucose) without exception are normally found to be present even in adult animals in the aqueous in lower concentration than in the blood. This is suggestive that any secretory process which can transfer ascorbic acid

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across the blood-aqueous barrier is relatively specific for this compound.

The question of the specificity of the transfer of ascorbic acid across the blood-aqueous barrier is of theoretical importance to a more complete understanding of the secretory process in general. Friedenwald and co-workers[3] have shown that ascorbic acid, presumably by reason of its ability to become reversibly oxidized and reduced, acts as a mediator in the oxidation-reduction system associated with the secretory process in general. It is not known, however, whether any portion of the molecule other than that concerned with oxidation reduction, that is, the enediol, is necessary either for secretion of other components of the aqueous humor or for the secretion of ascorbic acid itself. It would appear that this question could be resolved by studying the rate of transfer across the blood-aqueous barrier of the enantiomorph of ascorbic acid, d-isoascorbic acid, and d-glucoascorbic acid. D-isoascorbic acid differs structurally only in configuration and d-glucoascorbic acid in the addition of an extra CHOH group between carbon atoms 5 and 6 of ascorbic acid. Both compounds incidentally appear to have oxidation-reduction properties identical with ascorbic acid.

Materials and Methods

Albino rabbits were used for all of the experiments. The adult animals were fed a stock diet of Purina rabbit pellets, and the young animals were housed with their mothers.

The test substances were dissolved in glass-distilled water and injected intraperitoneally. Samples were taken from the eye at predetermined times under topical anesthesia (tetracaine HCl) using micropipettes as described previously,[1] and blood was removed by heart puncture. Upon collection, the samples were immediately placed in 4-percent metaphosphoric acid.

All of the analyses were done by means of the dichlorophenol-indophenol titration, and the results of all three compounds tested are reported in terms of ascorbic-acid equivalents. Since the method did not differentiate between the ascorbic acid normally present in the aqueous humor or blood and the compounds injected, the results must be interpreted in terms of change in concentration from the normal level. For the aqueous humor, the normal level is about 20 to 25 mg. percent; and for the blood, about 0.5 to 2.0 mg. percent.

Results

As to the manner in which ascorbic acid is transferred, Figure 1 shows that the

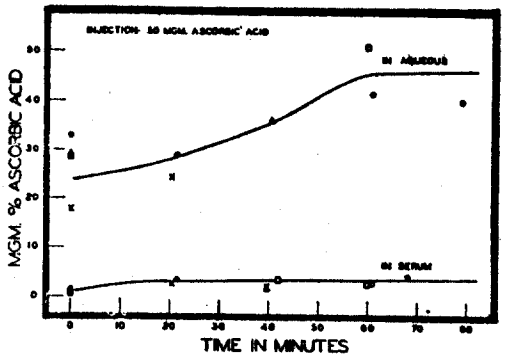

Fig. 1 (Kinsey). Shows the change in concentration of ascorbic acid in the serum and aqueous humor at various intervals following intraperitoneal injection.

concentration of ascorbic acid reaches a maximum in the blood within 20 minutes and that the concentration in the aqueous humor also increases to reach a maximum 60 minutes following intraperitoneal injection. Since the elevated level of ascorbic acid in the aqueous humor is maintained as long as the blood level remains constant, it may be assumed that a steady state is reached at this time.

Figure 2 shows the concentration of ascorbic acid in the aqueous 60 minutes after the injection of various quantities of this compound plotted as a function of the concentration in the blood. It is evident from the graph that the concentration in the aqueous increases rapidly with

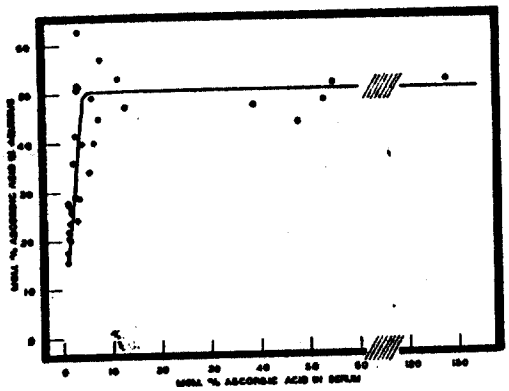

Fig. 2 (Kinsey). Shows the relative concentration of ascorbic acid in the aqueous humor and in the serum of adult rabbits.

increasing concentration in the serum until the level in the aqueous becomes 50 mg. percent. This occurs when the concentration in the serum is 3 mg. percent. With higher blood levels, no further increase in the quantity of ascorbic acid in the aqueous humor was observed, even though the concentration in the serum was elevated to 130 mg. percent.

The same experiments were repeated using young rabbits and the results are represented by the open circles in Figure 3. In this instance it will be seen that the concentration in the aqueous humor rises with increased concentration in the blood throughout the entire range. These results appear to be consistent with the hypothesis previously set forth; namely, that in adult animals ascorbic acid is transferred by secretory process, that some factor other than availability of this compound in the blood limits the amount which can be transferred across the blood-aqueous barrier, and that in young animals the transfer takes place primarily by the process of diffusion. That the blood-aqueous barrier in adult animals is impermeable to the simple diffusion of ascorbic acid is borne out by the observation that the concentration of ascorbic acid in the aqueous humor does not increase beyond 50 mg. percent, even though the blood level is more than double this concentration. It is interesting to note that molecules considerably larger than ascorbic acid—for example, sucrose—apparently can diffuse through the blood-aqueous barrier. The distinguishing characteristic between ascorbic acid and molecules like glucose and sucrose seems to be that, at the hydrogen-ion concentration found in the aqueous, ascorbic acid is charged; whereas, the sugars are not. This finding offers further support to the theory set forth by the author elsewhere[4] that electrolytes in general enter the eye by secretion; whereas, nonelectrolytes enter by diffusion.

With regard to the problem of the specificity of the secretory process, Figures 4 and 5 show the results of analyses made on serum and aqueous humor at various times after injecting d-iso ascorbic and

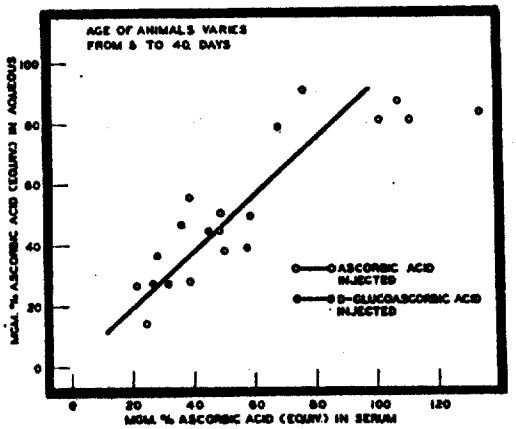

Fig. 3 (Kinsey). Shows the relative concentration of ascorbic acid (open circles) and d-gluco ascorbic acid (filled circles) in the aqueous humor and serum of young rabbits.

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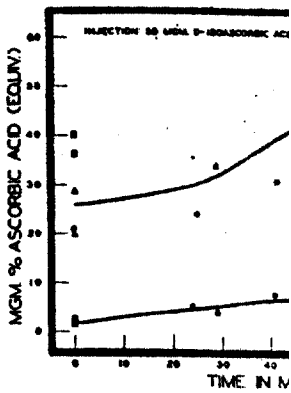

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d-gluco ascorbic acid into adult rabbits. In both instances the concentration of material titratable with 2:6 dichlorophenol indophenol (expressed as mg. percent ascorbic-acid equivalent) increased in the serum, but only in the case of d-iso ascorbic acid did the concentration of the injected material rise in the aqueous humor.

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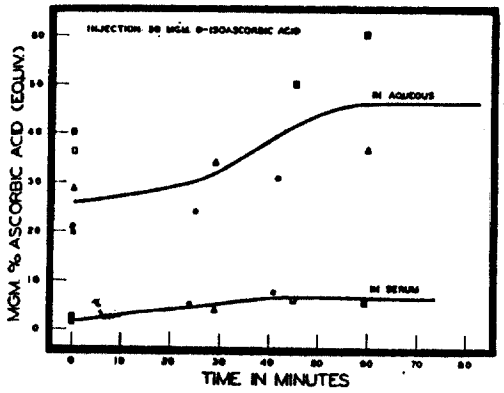

Fig. 4 (Kinsey). Shows the change in concentration of d-iso ascorbic acid in the serum and aqueous humor at various intervals following intraperitoneal injection.

concentration of d-gluco ascorbic acid in the serum and aqueous humor of young rabbits. Unlike the results obtained in the adult animals, it will be seen that d-glucoascorbic acid enters the aqueous humor of young animals and does so in proportion to the concentration in the serum.

The results indicate that the secretory process is apparently selective for ascorbic acid or its optical isomere and are suggestive that ascorbic acid forms an integral part of the secretory mechanism as proposed by Friedenwald.[3] The secretory process is not as specifically dependent upon a particular structure, however, as are the mechanisms whose malfunction results in scurvy, since d-isoascorbic and d-glucoascorbic have but 1/10 and 1/100 antiscorbutic power of ascorbic acid, respectively.

From the point of view of chemical mechanism, it may be inferred that in addition to the possible participation of that portion of the molecule responsible for the oxidation-reduction property; that is, the enol groups, the part of the molecule around carbon-atoms 5 and 6 are also necessary to the secretory process.

Summary

The distribution of ascorbic acid in the aqueous humor and blood of adult and young rabbits was determined under steady-state conditions. In adult animals the concentration of ascorbic acid in the aqueous was found to increase rapidly with increasing concentration in the blood until the concentration in the aqueous reached 50 mg. percent. This occurred with a serum concentration of approximately 3 mg. percent. With higher blood levels no further increase occurred in the concentration of ascorbic acid in the aqueous even though the concentration in the bloood was more than double that in the aqueous humor.

In young animals the concentration of ascorbic acid in the aqueous was found to vary directly with the concentration in the blood. These differences in distribution

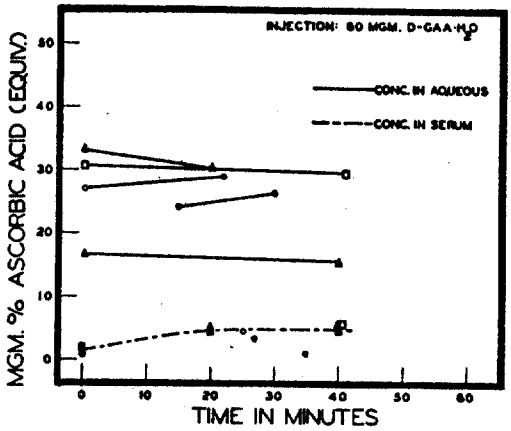

Fig. 5 (Kinsey). Shows the change in concentration of d-gluco ascorbic acid in the serum and aqueous humor at various intervals following intraperitoneal injection.

were thought to indicate that ascorbic acid enters the aqueous humor as a result of a secretory process in adult rabbits; whereas, in young animals the predominating factor in determining the distribution ratio is diffusion. Furthermore, they show that in adult animals some factor, other than availability of this compound in the blood, limits the amount which can be secreted across the blood-aqueous barrier.

The specificity of the secretory process for compounds structurally like ascorbic acid was investigated by injecting d-iso and d-gluco ascorbic acid into the peritoneal cavity and determining the rate of entrance of these compounds into the aqueous humor in adult and young rabbits. It was found that d-iso ascorbic acid entered the aqueous but no transfer of d-gluco took place across the blood-aqueous barrier in adult animals. These results indicate that the secretory process is apparently selective for ascorbic acid or its optical isomere (d-isoascorbic acid). The selectivity appears to be unrelated to the antiscorbutic action of these compounds. No selective transfer was observed for these compounds in eyes of young animals.

243 Charles Street (14).

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ervoirs containing identical fluids. The resistance of the membrane to both forward and reverse currents was measured, and its linearity examined. The measured membrane potential and resistance enabled calculation of the membrane short-circuit current as well as the electrical work done. The membrane power was calculated and its possible correlation with secretory activity discussed.

1430 Tulane Avenue (12).

ACKNOWLEDGMENT

The authors gratefully acknowledge the support, stimulation, and advice of Dr. James H. Allen, Professor and Chairman of the Department of Ophthalmology, Tulane University School of Medicine.

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THE EFFECT OF HYPOTHERMIA ON AQUEOUS HUMOR DYNAMICS

III. TURNOVER OF ASCORBATE AND SODIUM

BERNARD BECKER, M.D.*
St. Louis, Missouri

Systemic hypothermia in rabbits reduces the rate of aqueous secretion as determined by tonometric, tonographic and perfusion studies.[1] Flow declines exponentially with the decrease in body temperature. At 19°C secretion is estimated by these physical methods to be reduced to some 10 to 20 percent of the normothermic rate. It was the purpose of the present studies to measure the turnover of ascorbate and sodium in the aqueous humor of hypothermic rabbits in order to estimate the rate of aqueous flow.

METHODS

Hypothermia was induced in two to three kilogram male albino rabbits by immersion in an ice bath, using the short acting barbiturate, thiamylal sodium (Surital†), as necessary to prevent shivering during the induction period. Rectal temperatures were measured at regular intervals and room temperature was maintained at 20°C. Animals could be maintained at temperatures as low as 18-20°C for 5-8 hours.

The turnover of sodium[24] was carried out on 30 normothermic and 47 hypothermic animals at a mean (± S.D.) temperature of 19.2 (± 1.1)°C. Injections of approximately 50 μc were made (one-third intravenously and two-thirds intraperitoneally so as to maintain a constant blood level). Samples of posterior chamber and anterior chamber aqueous humor as well as plasma were taken at various time intervals, air dried and counted in a proportional gas counter. Re-

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† Provided by Parke, Davis and Company.

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sults were analyzed by linear (X-Y) methods.

The formulation used for these purposes was as follows:[2,3]

$$\frac{dC_a}{dt} = k_{fa}C_h + k_{dpa}(C_p - C_a) - k_{fa}C_a$$

where

$\frac{dC_a}{dt}$ = the rate of change of concentration of the solute in the anterior chamber aqueous

k_{fa} = the rate of aqueous flow per minute as a fraction of the anterior chamber volume

k_{dpa} = the rate of diffusional exchange per minute of the solute between anterior chamber and plasma (as a fraction of the anterior chamber volume)

C_p = concentration in arterial plasma in relative units or mg percent

C_a = concentration in anterior chamber aqueous in relative units or mg percent

C_h = concentration in posterior chamber aqueous in relative units or mg percent

Dividing both sides of the equation by $(C_p - C_a)$:

$$\frac{dC_a}{dt} \Big/ (C_p - C_a) = k_{fa}\frac{(C_h - C_a)}{(C_p - C_a)} + k_{dpa}$$

where

$$Y = k_{fa}X + k_{dpa}$$

$$Y = \frac{dC_a}{dt} \Big/ (C_p - C_a)$$

$$X = \frac{C_h - C_a}{C_p - C_a}$$

Plotting X versus Y at different time intervals provided two separate straight lines corresponding to the normothermic and hypothermic data. For each of these lines the slope estimates k_{fa} and the Y-intercept k_{dpa}.

The turnover of ascorbate in 15 hypothermic animals (19.8 ± 0.9°C) was attempted at plasma levels of 10 mg percent by the administration of 50 mg of sodium ascorbate intravenously.[4] When this proved unsuccessful as a method of measuring turnover, plasma levels averaging 160 mg percent were achieved in 60 hypothermic rabbits (19.1 ± 1.0°C) by the single intravenous injection of 1000 mg of sodium ascorbate. In addition 82 rabbits were "saturated" with ascorbate in the normothermic state by the administration of sodium ascorbate 100 mg intravenously and 100 mg subcutaneously. Two hours later plasma and aqueous samples from one eye of these normothermic rabbits were obtained. They were then subjected to hypothermia (19.5 ± 0.6°C) and the rates of change of ascorbate concentration in the posterior and anterior chambers were measured as functions of time. After hypothermia plasma levels were found to remain at approximately 10 mg percent in these experiments without need for further injection. Both of these ascorbate approaches provided data suitable for X-Y analysis. Ascorbate was determined on all samples by delivery into 4 percent metaphosphoric acid and titration immediately with dichlorphenolindophenol, using a 0.1 ml Gilmont ultramicroburet.

All posterior chamber and anterior chambers fluids were withdrawn from the proptosed eyes into calibrated pipets. In hypothermic rabbits it was found necessary to use 27 gauge needles for posterior chamber taps in order to avoid vitreous contamination. Bloods were obtained by cardiac puncture using a heparinized syringe. Animals were used once and then discarded.

RESULTS

1. *Turnover of radioactive sodium.* The turnover of sodium^{24} in the rabbit eye was plotted as a function of time (fig. 1). At each time interval the mean value ± S.D. for 10 to 20 eyes was used. The values determined simultaneously in normothermic

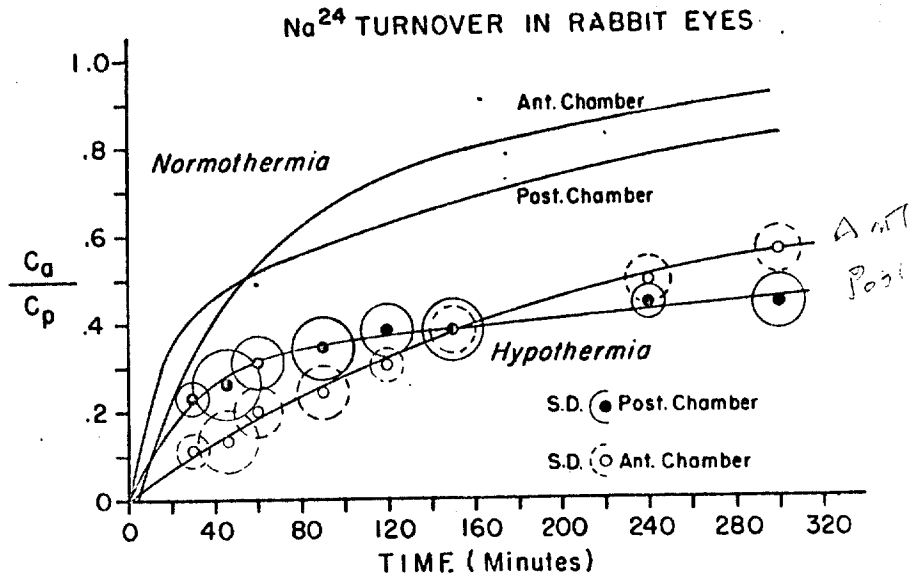


Fig. 1 (Becker). Relative concentrations of radioactive sodium in posterior and anterior chamber aqueous humors of hypothermic rabbits at various times after injection. At each time the mean value (± S.D.) for 10 to 20 animals is presented. Normothermic values drawn for comparison. C_a = concentration in aqueous humor, C_p = concentration in plasma water.

rabbits agreed well with those obtained previously. They are plotted in Figure 1 for comparison.[5] The posterior and anterior chamber concentrations in the hypothermic animals at various times provided curves that were of similar shape to that in normothermic animals. It was apparent that the accumulation of radioactive sodium in both chambers of hypothermic animals was slower, and that the crossover point of the

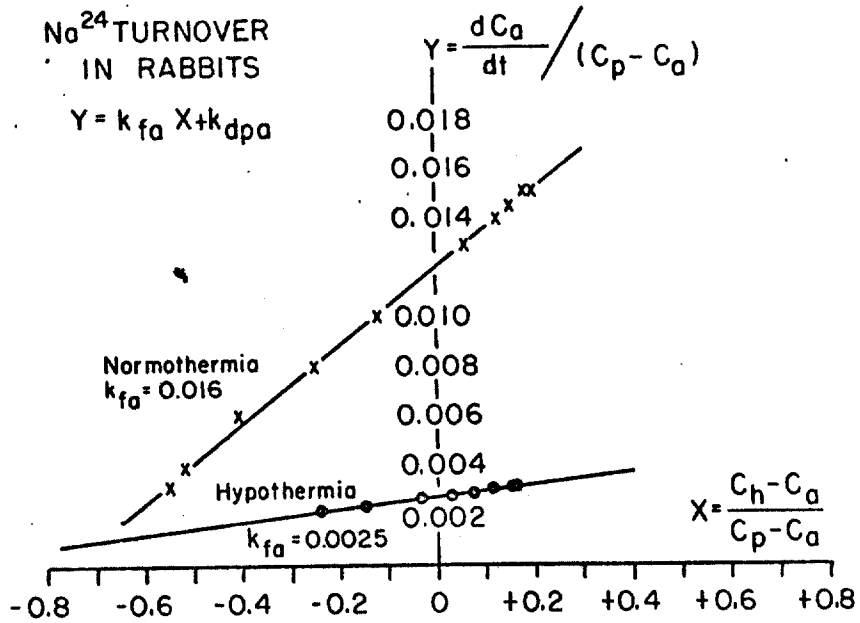


Fig. 2 (Becker). Linear (X-Y) analysis of sodium turnover in normothermic and hypothermic rabbits. The slope of each line estimates k_{fa}, the flow coefficient.

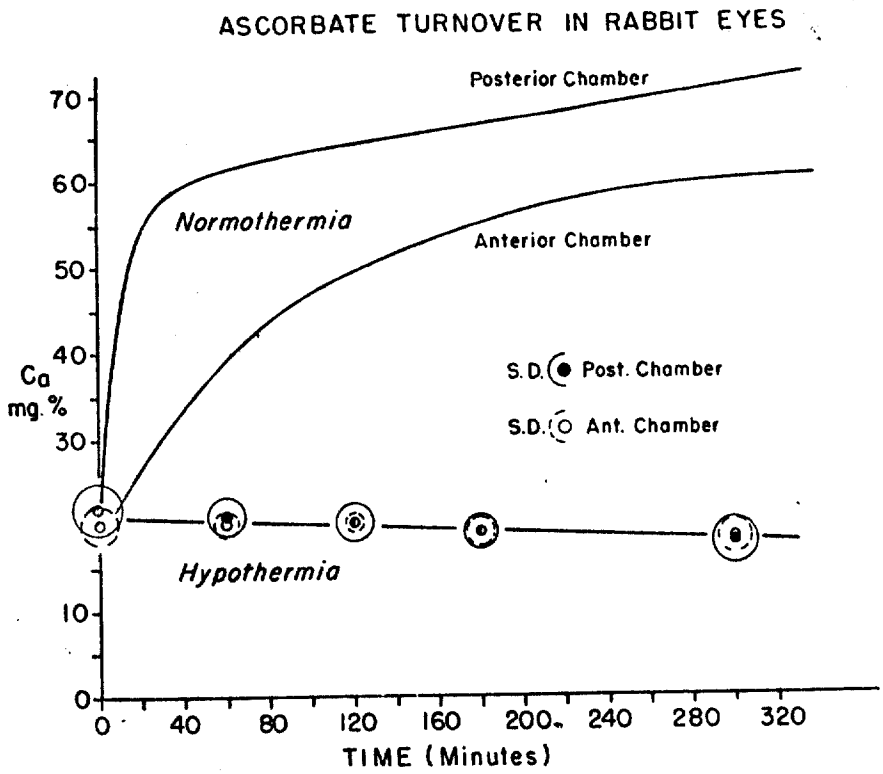


Fig. 3 (Becker). Concentration of ascorbate (C_a) in aqueous humor of posterior and anterior chambers of hypothermic rabbits at various times after the intravenous injection of sodium ascorbate. Plasma levels of 10 to 15 mg percent were maintained. The responses to this plasma level in normothermic rabbits are presented for comparison.

anterior and posterior chamber curves occurred later and at a lower concentration.

X-Y plots (fig. 2) of the posterior and anterior chamber data were analyzed and compared with normothermic rabbits. It was apparent that the rate of flow was reduced from the normal level of 1.6 percent of the anterior chamber per minute to 0.25 percent per minute at 19°C (a reduction to approximately 16 percent of the normothermic value). In addition the diffusion coefficient was decreased by hypothermia to 0.28 percent per minute from a normal of 1.2 percent per minute.

2. *Turnover of ascorbate.* In the normothermic animal the maintenance of a plasma concentration of 10 to 15 mg percent of ascorbic acid results in high concentration levels in the posterior and anterior chamber fluids. The rate of approach to these levels has been used as a means of measuring aqueous flow.[5] Attempts to utilize the same method in the hypothermic animal proved unsuccessful. In spite of a plasma level of 10 mg percent, posterior and anterior chamber ascorbic acid levels failed to rise, and in fact both declined slowly. Comparison of the normal and hypothermic responses to a plasma level of 10 to 15 mg percent of ascorbic acid are presented in Figure 3.

In order to alter aqueous ascorbate concentrations it was necessary to elevate the plasma ascorbate level considerably above the levels in the aqueous humor. This was accomplished by intravenous injection of 1000 mg of sodium ascorbate. In the hypothermic animals average blood levels of 160 mg percent resulted. Under these circumstances the concentrations in the posterior and anterior chamber aqueous humor rose slowly. In Figure 4 the aqueous humor concentrations for 10 to 20 eyes at each time

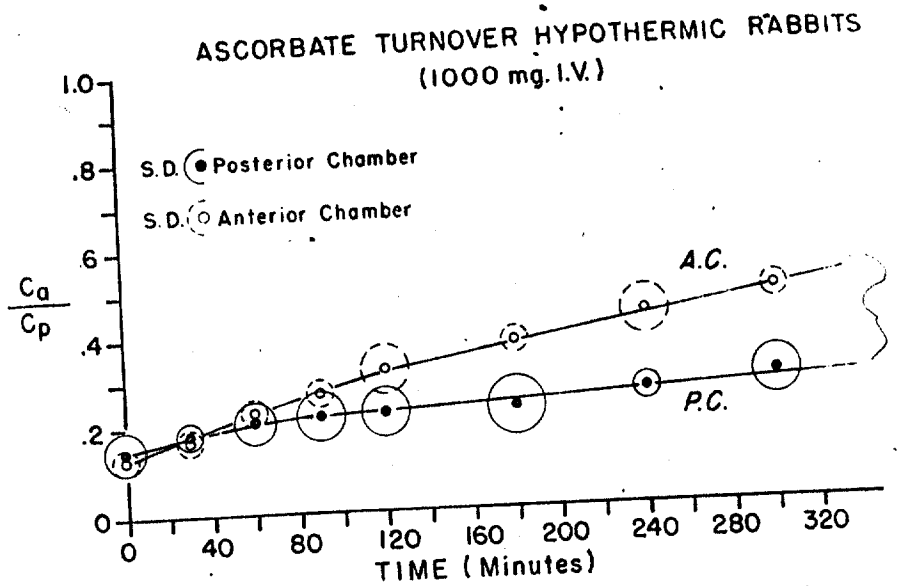


Fig. 4 (Becker). Aqueous humor concentrations of ascorbate in hypothermic rabbits at various time intervals after the intravenous injection of 1000 mg of sodium ascorbate. Plotted are the mean values (± S.D.) for aqueous humor (C_a) as a fraction of the plasma level (C_p) for 10 to 20 eyes at each time.

interval are plotted as a fraction of the plasma concentration (mean ± S.D.) against time (minutes). It is apparent that the anterior chamber concentrations rose more rapidly than did those of the posterior chamber.

X-Y analysis (fig. 5) of the ascorbate data suggested a flow rate of 0.20 percent of the anterior chamber per minute at 19°C. compared with previous estimates of 1.5 to 1.7 percent per minute in the normothermic animal (or a reduction to 12 to 13 percent of normal at this hypothermic level). The diffusion coefficient was reduced from the nor-

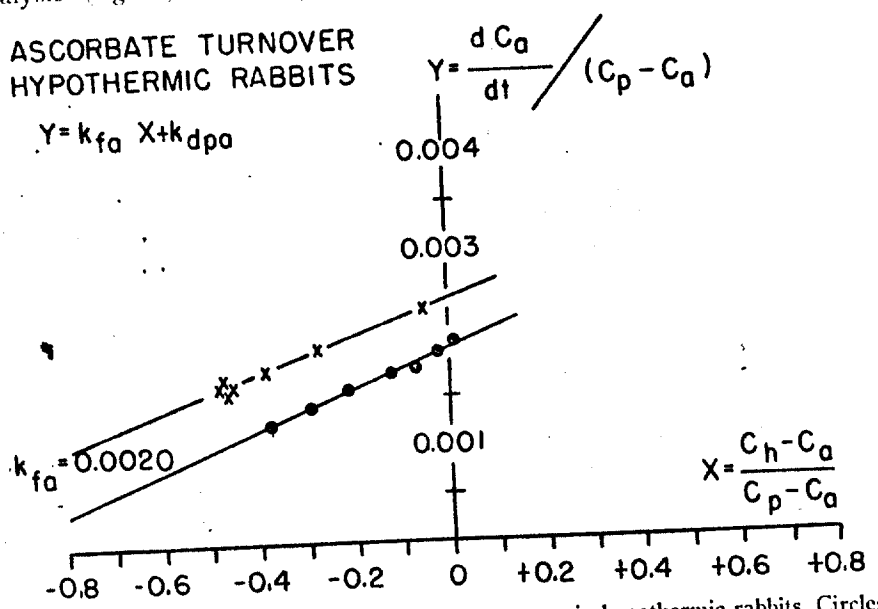
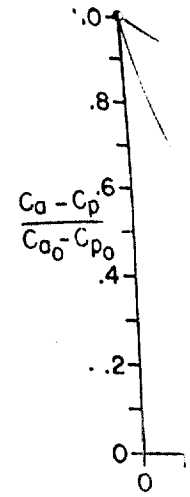


Fig. 5 (Becker). Linear (X-Y) analysis of ascorbate turnover in hypothermic rabbits. Circles represent data from intravenous injections of 1000 mg in hypothermic animals (see fig. 4); crosses are similar analysis of data of Figure 6. Slopes of lines provide estimates of k_{fa}, the flow coefficient.



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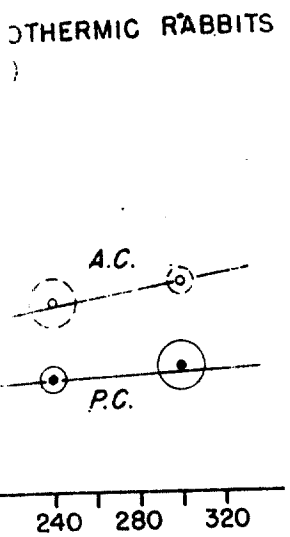

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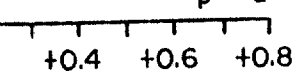

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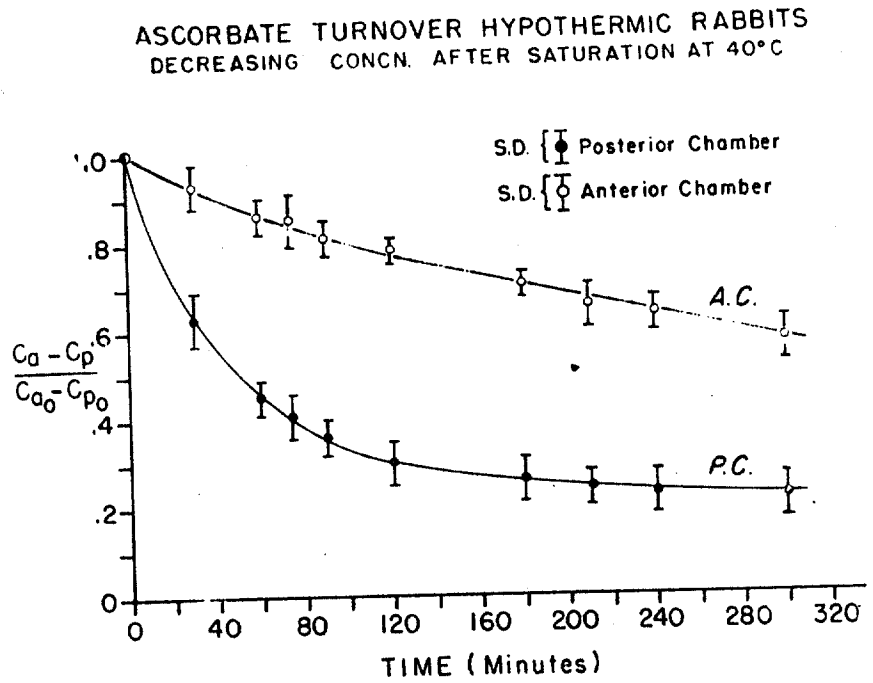

Fig. 6 (Becker). Rate of decline in aqueous humor ascorbate concentrations following hypothermia in the ascorbate saturated animal (sodium ascorbate 100 mg intravenously and 100 mg subcutaneously to normothermic animals two hours before hypothermia). Ascorbate excesses over plasma (C_a-C_p) for posterior chamber and anterior chamber are plotted as fractions of pre-hypothermic values (mean ± S.D. for 10 to 15 eyes at each time interval).

ual of 0.5 percent per minute to 0.20 percent per minute at 19°C.

Another approach to ascorbate turnovers was provided by the administration of ascorbate 100 mg intravenously and 100 mg subcutaneously two hours before hypothermia was induced. This resulted in posterior chamber values averaging 66 percent, anterior chamber values of 50 mg percent, and plasma values of approximately 10 mg percent just prior to the time hypothermia was induced. One could then follow the time course of the fall in posterior and anterior chamber concentrations of ascorbic acid under hypothermia with a plasma level maintained at approximately 10 mg percent. These values were plotted as the mean (± S.D.) concentrations of ascorbate in excess of plasma in the aqueous humor of hypothermic (19.5°C) animals as a fraction of the values in excess of plasma before hypothermia (6 to 14 eyes at each time interval). This method of plotting the data (fig. 6) tended to minimize the variation among animals and provided suitable curves for X-Y analysis. Under the circumstances of these experiments the posterior chamber concentrations fell much more rapidly than did those of the anterior chamber. This may well be related to the adjacent large volume of vitreous which had failed to become "saturated" in the brief two hour normothermic period. X-Y analysis of the data revealed a flow coefficient of 0.20 percent of the anterior chamber per minute and a diffusion coefficient of 0.25 percent of the anterior chamber per minute (fig. 5).

Discussion

In Table 1 the results of the measurement of flow by various methods used on hypothermic rabbits are summarized and compared with the same techniques in normal and acetazolamide treated animals.[5] For purposes of this comparison the volume of

TABLE 1

COMPARISON OF RATE OF AQUEOUS FLOW* IN NORMAL, HYPOTHERMIC, AND ACETAZOLAMIDE TREATED RABBITS

Method	Normal	Hypothermia (19°C)	Acetazolamide
Ascorbate	4.3	0.5	2.1
Na^{24}	4.0	0.6	1.8
Tonography	4.1	0.6	1.9

* Mean values (μl./min.) assuming volume anterior chamber of 250 μl.

the anterior chamber is assumed to average 250 μl and rate of flow is expressed in μl/min. In animals at 19 to 20°C tonography as well as turnover studies with sodium and ascorbate demonstrate that the rate of flow is reduced to 10 to 20 percent of normal. Similar measurements following acetazolamide administration demonstrated that flow is reduced to 45 ot 55 percent of normal. As in the case of carbonic anhydrase inhibition there is excellent agreement of the estimates of altered flow by diverse methods. In contrast to the acetazolamide effects which are confined to flow, however, hypothermia appears to also decrease the rate of diffusion of various test substances.

Unfortunately since flow and diffusion coefficients are both altered by hypothermia, it is not possible to use alterations in steady state concentrations as measures of change in flow rate for these animals. The use of this method presents further difficulties because of the enormous time interval needed to establish a new steady state when turnovers are so markedly reduced. There appears to be a tendency in the hypothermic rabbit, however, for the ascorbate concentration of the aqueous humor to fall and the bicarbonate concentration to rise.

Although it may be reasonably accepted that hypothermia decreases the rate of aqueous humor formation, the intimate mechanisms of this effect remain obscure. The decrease in aqueous flow may reflect a reduction in blood flow to the eye resulting from the decreases in cardiac output, pulse, and blood pressure, the increase in viscosity of blood, and possible local vasoconstriction induced by cold. Unfortunately the marked suppression of secretion of ascorbate into the aqueous humor precludes the use of this method of estimating blood flow in the ciliary body.[6] It is also conceivable that the decrease in rate of aqueous secretion may be a consequence of effects of hypothermia on the central nervous system, on various endocrine glands, on circulating metabolites, or more directly on the metabolism and secretory activity of the ciliary epithelium. Experimental approaches are needed to evaluate the role of each of the above factors in the overall process.

Alterations in diffusion coefficients for various substances also need further study. A part of the changes observed may result from such known effects of cooling as the reduction in rate of diffusion of solutes, alterations in solvent viscosity, and alterations in diameter of blood vessels. The temperature dependence of the metabolic state of living membranes and the active processes which maintain these barriers are also likely to be major factors in the alterations of the diffusion coefficients.

SUMMARY

In rabbits immersion hypothermia to 19°C has been reported to reduce the rate of aqueous flow to 10 to 20 percent of normal as measured by repeated tonometry and tonography. The turnovers of radioactive sodium and of ascorbate in the eyes of such hypothermic rabbits provided data compatible with this degree of secretory suppression. Thus, the coefficient of aqueous flow was reduced from the normothermic value of 1.6 percent of the anterior chamber per minute to 0.25 percent of the anterior chamber per minute at 19°C, as measured with sodium[24]. The comparable values derived from ascorbic turnover were 1.[illegible] percent normothermic and 0.20 percent at 19°C.

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† From the Department
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Summary

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THE EFFECT OF HYPOTHERMIA ON AQUEOUS HUMOR DYNAMICS

IV. Carotid Artery Ligation and Blood Flow*

Irvin P. Pollack, M.D.†‡ and Bernard Becker, M.D.†

St. Louis, Missouri

Aqueous flow is decreased following immersion hypothermia in rabbits.[1, 2] The present study attempts to determine whether there are changes in blood flow through the ciliary body during hypothermia and their possible relation to alterations in the rate of aqueous secretion. Unilateral carotid ligation and preganglionic sympathectomy are utilized in order to modify the alterations in ocular blood flow induced by hypothermia.

Methods

The common carotid artery on one side was isolated, ligated, and cut under a short-acting general anesthesia, thiamylal sodium,§ in 57 male albino rabbits weighing 2 to 3 kg. The contralateral eye was maintained as a control. Eighteen to 24 hours later immersion hypothermia was induced in an ice bath in the manner previously described.[1] Room temperature was maintained at approximately 20°C.

Tonography or tonometry was performed on both eyes prior to carotid ligation as a control. It was repeated 18 to 24 hours after the operation, before and during hypothermia. An electronic tonometer was used with a Leeds and Northrup recorder. Topical tetracaine (0.5 percent) was used for those rabbits studied at normal temperatures; however, it was not required for those animals studied under hypothermia. All scale readings were interpreted according to Wistrand's calibration curve for the rabbit.[3] Aqueous flow rates were calculated from the equation $F = C(P_o - P_v)$ assuming an episcleral venous pressure of 9 mm Hg.

Unilateral preganglionic sympathectomy was performed on 12 rabbits. The effectiveness of the procedure was determined by

* This investigation was supported in part by a research grant, B-621, from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service. The research relating to this study was also financed in part under a grant to Washington University School of Medicine made by the Alfred P. Sloan Foundation, Inc. The grant was made upon recommendation of the Council for Research in Glaucoma and Allied Diseases. Neither the Foundation nor the Council assumes any responsibility for the published findings of this study. Read at the Midwinter National Meeting of the Association for Research in Ophthalmology, New Orleans, Dec. 5, 1960.

† From the Department of Ophthalmology, Washington University School of Medicine and the Oscar Johnson Institute, St. Louis, Missouri.

‡ Presently at The Wilmer Institute, Johns Hopkins Hospital, Baltimore 5, Maryland.

§ The thiamylal sodium (Surital sodium) was generously provided by Parke Davis.

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INTRAVENOUS GLYCEROL-VITAMIN C (SODIUM SALT) AS OSMOTIC AGENTS TO REDUCE INTRAOCULAR PRESSURE

MICHELE VIRNO, M.D., MASSIMO G. BUCCI, M.D., JOSE PECORI-GIRALDI, M.D., AND GIAMPAOLO CANTORE, M.D.
Rome, Italy

The truly remarkable osmotic effect in lowering intraocular pressure displayed by oral glycerol was first reported by us in 1962[1,2,4] and recently confirmed by numerous other authors.[5-24]

Because of its effectiveness, at the present time oral glycerol is preferred in most cases to the other osmotic agents (urea, mannitol) whose administration is necessarily intravenous.

In some cases, however, due to clinical conditions and to the individual status of the patient (already under general anesthesia or predisposed to vomiting), the oral glycerol is not used and intravenous administration of an osmotic agent is required.

Among the intravenous osmotic ocular hypotensive agents, mannitol has proven useful, the side-effects following its use being less than those of urea,[18,19] although its action is not so marked.

Yet, the large volume of mannitol solution (approximately 800 ml) and the dosage (2.0-2.5 gm/kg body weight, intravenously) required to obtain the ocular hypotensive effect and the length of time needed to reach the maximum effect represent great disadvantages.

In view of the prompt and marked ocular hypotensive action of oral glycerol, it seems reasonable to assume that, if intravenous administration of the drug could be made possible, a much greater hypotony of the ocular globe could be induced. At the same time, the volume of solution to be infused could be reduced and, consequently, the infusion time shortened.

Recent studies[25] on the clinical use of intravenous glycerol showed that, by this route, the drug, as a 30% solution in saline in doses of 250 mg/kg up to 2.0 gm/kg body weight, produced hematuria both in the experimental animal and in man.

The histologic preparations of the kidneys of rabbits, killed at different time intervals (15 and 45 minutes; 3, 7 and 12 hours) following administration of 2.0 gm/kg body weight of 30% glycerol in saline, showed that the hematuria was due to a transient functional alteration of the afferent glomerular arterioles.

Immediately after the introduction of glycerol, a block of diuresis lasting 30 to 40 minutes was observed in the catheterized animals. Throughout this period, there was a vasoconstriction of the afferent glomerular arterioles, evidenced by numerous areas of subcapsular ischemia. A reflex vasodilatation followed.

The glomerular loops were found to be swollen and occupied the entire inner capsular space. Intracapsular and interstitial hemorrhages were also noted. The vasodilatation lasted five to eight hours, allowing the passage of blood by rexin and diapedesis from the glomerular filter. Twelve hours later, the renal alterations had disappeared and the morphologic pattern of the kidney was found to be completely normal.

In a subsequent work,[26] we reported that the only substance among those tested capable of completely eliminating the occurrence of anuria, hematuria and of the renal histologic alterations produced by intravenous 30% glycerol in saline[25] was the sodium salt of vitamin C—sodium ascorbate. This drug can protect against the effects produced on the kidney by intravenous glycerol through immediate osmotic diuresis when adminis-

From the Eye Clinic of the University of Rome (Director: Prof. G. B. Bietti), the Faculty of Medicine, University of Rome, and the Clinica delle Malattie Nervose e Mentali, University of Rome.

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The pharmacologic effects of sodium ascorbate, injected in high doses both in the experimental animal and in man, were studied by Virno and co-workers,[27] who first described that, as a 20% solution in water at pH 7.2 to 7.4, in doses of 1.0-1.5 gm/kg of body weight, this drug was one of the most effective osmotic agents in reducing intraocular and intracranial pressure.

This paper reports further studies on the ocular hypotensive effects obtained, both in experimental animals and in man, with intravenous sodium ascorbate and with intravenous glycerol combined with sodium ascorbate. Two preliminary reports[28,29] described the effects in a limited number of patients suffering from various types of intraocular hypertension.

Methods

In all trials, both experimental and clinical, vitamin C (pure crystal ascorbic acid, Merck 500074) was dissolved in bidistilled water to make a 20% solution which was titrated to pH values of 7.2 to 7.4 with sodium bicarbonate (Merck 6329).

Since the aqueous solution of vitamin C, at the pH we employed and in the presence of oxygen, is likely to oxidize with the passage of time, the solutions were used within 48 hours after preparation. We avoided the use of stabilizers because they could have altered the results.

It seemed that, by lyophilizing the solution of sodium ascorbate, it should be possible to obtain a stable product which, with the addition of bidistilled water, could make a 20% solution ready for clinical use (as with urea).

As for the trials carried out with combined glycerol and sodium ascorbate, we proceeded as follows: to 30 ml of pure glycerol were added 70 ml of the 20% vitamin C solution, titrated to pH 7.2 to 7.4 with sodium bicarbonate, to make a 100 ml solution. The final concentration of the combined substances was 44%.

Animal studies

Twelve male rabbits weighing 2.5 to 3.0 kg were used. In all animals, an indwelling vesical catheter was applied. Urine collection periods were before treatment, and, after treatment, at 30-minute intervals for nine hours beginning when the drugs were administered.

Urines were tested for the presence of blood, both by a chemical method (Mayer) and by microscopic examination (urinary sediment obtained by centrifugation).

In two groups of six rabbits each, intraocular hypertension was induced by the procedure described by Samis.[8] In order to perform tonometric readings more conveniently, the animals were immobilized with curare (gallamine triethiodide) and artificial pulmonary respiration was applied. Aqueous humor was withdrawn from the anterior chamber of the eye by inserting a 20-gauge needle obliquely through the cornea. Without removing the needle, the anterior chamber was filled with a 2.5% methylcellulose solution. The angle of the anterior chamber was obstructed by the methylcellulose impeding the outflow of fluids from the eye and, thus, an intraocular hypertension was obtained.

Intraocular pressure was measured with the Schiøtz tonometer (5.5 and 10 gm) and the readings were converted using the 1955 scale.

The solution was injected in the marginal vein of the ear of the rabbit at a rate of 5.0 ml per minute.

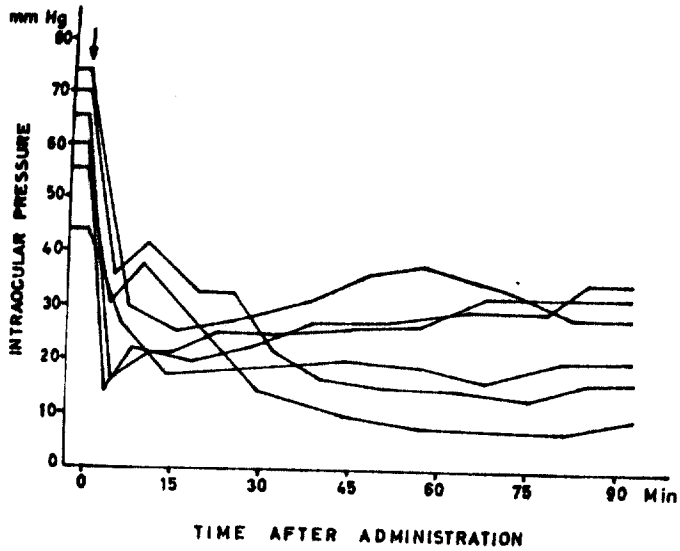

Fig. 1 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of intravenous sodium ascorbate on experimental glaucoma of six rabbits. At arrow: 1.5 gm/kg body weight of 20% sodium ascorbate was given intravenously.

Clinical studies

Solutions of 20% sodium ascorbate in bidistilled water at pH 7.2 to 7.4 and of combined 30% glycerol and 20% sodium ascorbate were sterilized through MF/10 membrane filters G D 15 (Membrane filter-Göttingen). Both solutions were administered within 48 hours after preparation at 80 to 100 drops per minute.

Besides taking the usual precautions (careful rinsing of all utensils with bidistilled water and sterilization of the solutions shortly after preparation), the rectal temperatures of rabbits given the solutions prepared for clinical use were measured to check for pyrogens.

Intraocular pressure was recorded at varying time intervals by a Schiøtz tonometer, using the 5.5 and 10 gm weights, and by an applanation tonometer.

In all patients, blood pressure was measured before, during and after infusion.

Urine was obtained from all patients and tested for the presence of blood by the method of Mayer.

Results

Experimental animals

Sodium ascorbate was administered intravenously to six rabbits, in whose right eyes intraocular hypertension had previously been induced by injecting a 2.5% methylcellulose solution. The average increase in intraocular pressure was to 54.80 mm Hg within about one hour. Once the intraocular hypertension was established, sodium ascorbate (1.5 gm/kg body weight) was given intravenously. Within five minutes after administration, the mean fall in intraocular pressure (fig. 1) was 38.80 mm Hg. Low tension levels were maintained for approximately two hours; then pressure slowly returned to pretreatment levels.

Tonometric tracings from the left eye of the same animals before administration of the drug indicated that the ocular tension was normal. Within five minutes following intravenous injection of 1.5 gm/kg of 20% sodium ascorbate, a decrease in intraocular pressure was observed. Mean tension fall was 18 mm Hg.

A combination of 30% glycerol and 20% sodium ascorbate was given intravenously to six rabbits. Intraocular pressure of the right eye of each animal had been increased by blocking the iridocorneal angle with a 2.5 methylcellulose solution. The combined solution (2.0 ml/kg body weight) was administered intravenously when mean tonometric values of 60 mm Hg were attained. Figure 2

Fig. 2 (Virno, Bucci, Giraldi and Cantore). Effect travenous association of and sodium ascorbate on mental glaucoma of six rabb arrow: 0.6 gm/kg body we 30% glycerol and 0.28 gm/k weight of 20% sodium as were given intravenously.

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Urines collected at following administration showed no hematuria (Mayer) or by microsc the urinary sediment gation.

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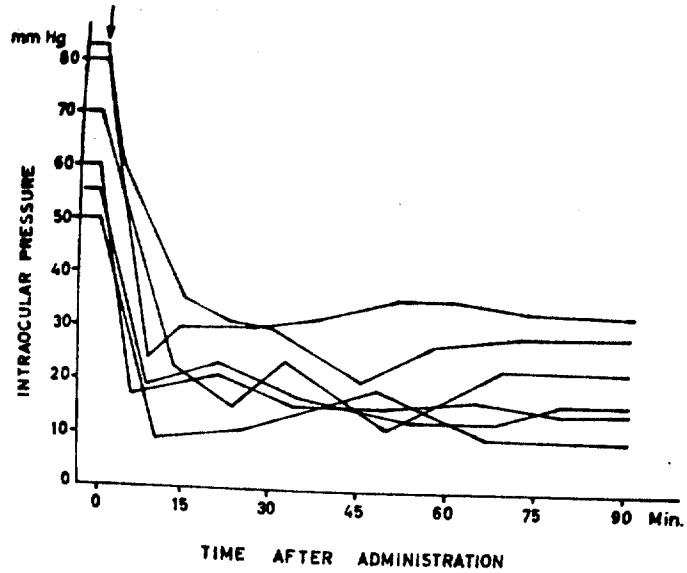

Fig. 2 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of intravenous association of glycerol and sodium ascorbate on experimental glaucoma of six rabbits. At arrow: 0.6 gm/kg body weight of 30% glycerol and 0.28 gm/kg body weight of 20% sodium ascorbate were given intravenously.

shows the reduction in pressure in the eyes of the six rabbits with experimental glaucoma, given 0.6 gm/kg body weight of 30% glycerol combined with 0.28 gm/kg body weight of 20% sodium ascorbate intravenously (that is, 2.0 ml/kg body weight of the combined medication).

Urines collected at varying time intervals following administration of both solutions showed no hematuria either by chemical (Mayer) or by microscopic examination of the urinary sediment obtained by centrifugation.

Clinical

I. The effects of intravenous sodium ascorbate were studied in five patients with glaucoma from the Ophthalmological Clinic of Rome University. Doses ranged from 0.4 to 1.0 gm/kg of body weight. The rate of administration was 80 to 100 drops per minute.

Of the nine eyes analyzed (one patient was one-eyed), two were suffering from chronic congestive glaucoma, one from secondary glaucoma, one from chronic simple (open-angle) glaucoma, one from buphthalmos (congenital glaucoma), one underwent an attack of acute glaucoma and three had normal intraocular pressure.

In all patients treated, sodium ascorbate caused marked reduction in intraocular pressure, attaining its maximum effect between 60 and 90 minutes from the start of the infusion.

Figure 3 shows the response to sodium ascorbate of a patient with chronic congestive glaucoma of the right eye. The patient, a 64-year-old woman, had previously undergone three uneventful antiglaucomatous surgical interventions. She has been referred because of failure to respond to acetazolamide (1.0 gm daily). Pretreatment pressure was 55 mm Hg, R.E., and 38 mm Hg, L.E. Intravenous sodium ascorbate (.04 gm/kg body weight) was administered and pressure was brought down to 21 mm Hg, R.E., and 10 mm Hg, L.E., within one hour from the start of the infusion. Low intraocular pressure was maintained for six hours.

Figure 4 shows the effect of intravenous sodium ascorbate on the intraocular pressure of a patient suffering from secondary glaucoma of the right eye and from chronic simple glaucoma of the left. Intraocular pressure had previously been kept under control by miotics. Within one hour after the intravenous administration of 0.5 gm/kg body weight of 20% sodium ascorbate at pH 7.2 to 7.4, there was a reduction in pres-

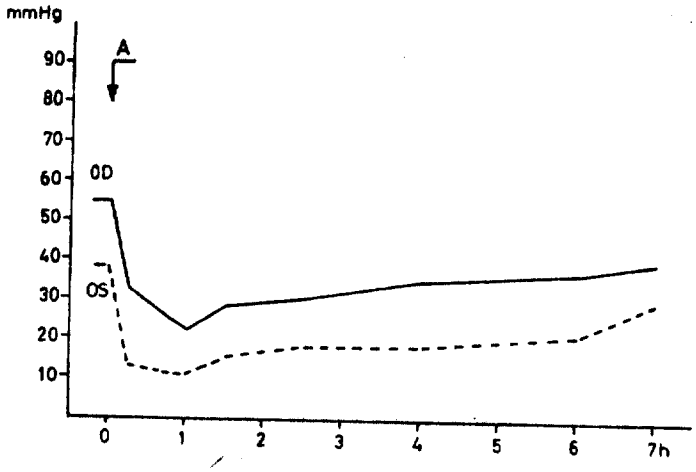

Fig. 3 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of intravenous sodium ascorbate on chronic congestive glaucoma of the right eye. At (A): 0.4 gm/kg body weight of 20% sodium ascorbate was given intravenously.

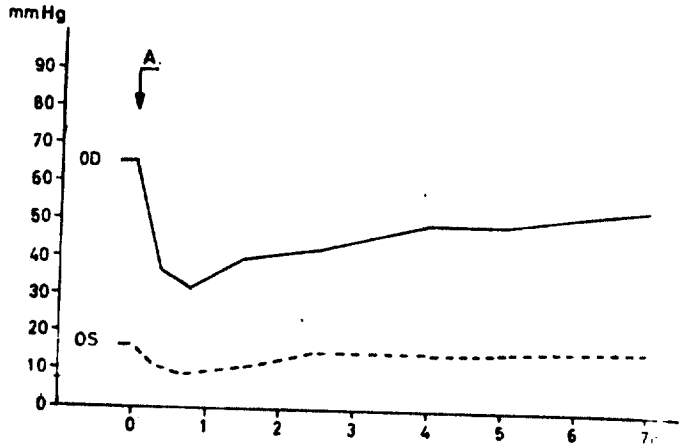

Fig. 4 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of intravenous sodium ascorbate on secondary glaucoma of the right eye. At (A): 0.5 gm/kg body weight of 20% sodium ascorbate was given intravenously.

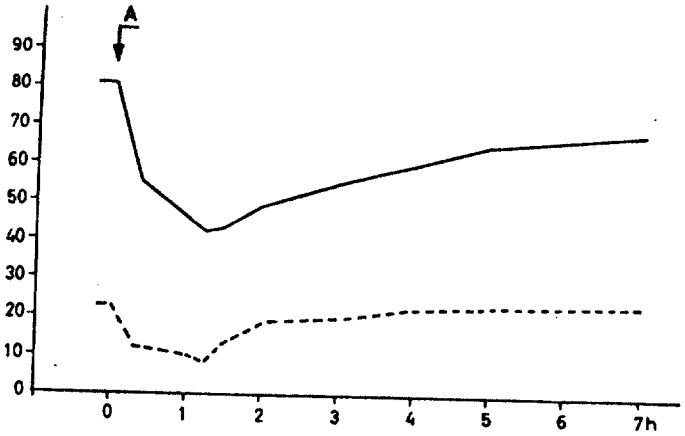

Fig. 5 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of intravenous sodium ascorbate on acute glaucoma of the left eye. Sodium ascorbate—0.5 gm/kg body weight intravenously—was given at (A) during an attack of acute glaucoma.

sure from 64 to 32 mm Hg, R.E., and from 17 to 8.0 mm Hg, L.E.

Figure 5 shows the reduction in pressure in an eye during an attack of acute glaucoma. Pretreatment pressure was 82 mm Hg, L.E. Intravenous sodium ascorbate (0.5 gm/kg body weight) was administered and tension fell to 41 mm Hg, L.E. Although the dosage employed was almost low with regard to the usual medications (urea:

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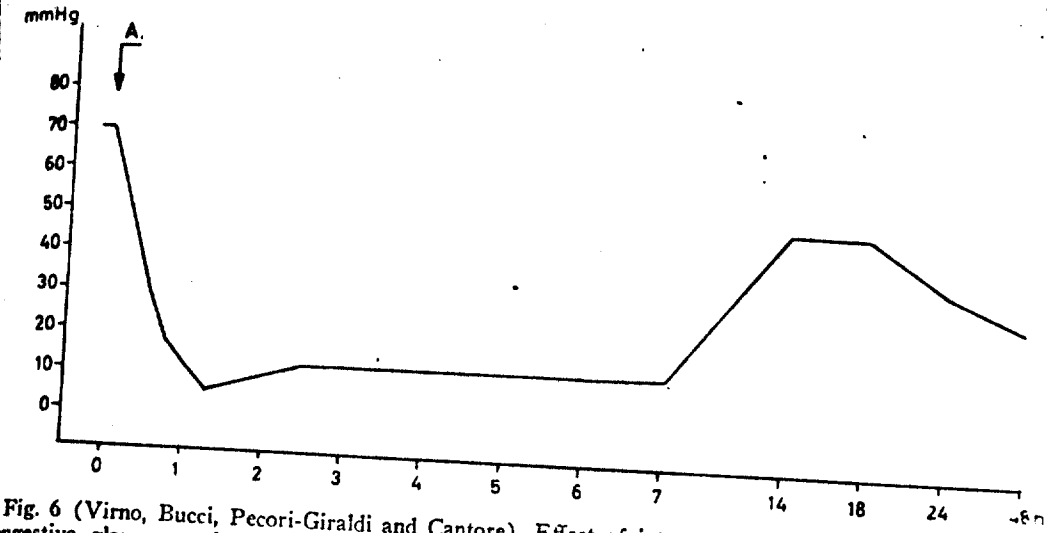


Fig. 6 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of intravenous sodium ascorbate on chronic congestive glaucoma of the right eye. Sodium ascorbate—1.0 gm/kg body weight intravenously—was given at (A) during an acute attack.

1.0-1.5 gm/kg body weight; mannitol: 2.0-2.5 gm/kg body weight) needed to obtain favorable responses, the drug has proven markedly effective. Intraocular pressure was promptly reduced in both eyes and, moreover, the pain and the decrease of visual acuity secondary to corneal edema in the left eye disappeared in a few minutes.

Figure 6 shows the ocular hypotensive effect obtained with 1.0 gm/kg body weight of 20% sodium ascorbate at pH 7.2 to 7.4 intravenously. The patient, a 45-year-old man, had been referred because of chronic congestive glaucoma in his only eye. The drug was administered during the time of an acute attack, although the patient did not experience any pain in his eye. No medical, topical or general management had been used previously. A maximum ocular hypotensive effect was attained within 70 minutes from the start of administration, with a reduction in pressure from 70 to 7.0 mm Hg. Tension levels of 12 mm Hg were maintained thereafter for approximately seven hours. Fourteen hours after the infusion, tension climbed to 52 mm Hg; four hours later, however, it fell again to 32 mm Hg, no drugs being administered.

In order to evidence its effectiveness as an osmotic agent to reduce intraocular hypertension, sodium ascorbate was directly compared with mannitol, one of the most commonly employed clinically useful osmotic agents.

The two tests were carried out on the same patient, a 22-year-old man suffering from buphthalmos (congenital glaucoma) of the right eye, who had been previously treated with pilocarpine three times daily. Administration of the two drugs was started when the pressure level was 43 mm Hg.

Intravenous 20% sodium ascorbate (1.0 gm/kg body weight) was given first and the pretreatment tension of 43 mm Hg on the right was brought down to 10 mm Hg in about 90 minutes.

On the following morning, tension had risen again to the same pretreatment level (43 mm Hg). Intravenous 20% mannitol (1.0 gm/kg body weight) was now administered and intraocular pressure was brought to 34 mm Hg 90 minutes later. The different tension-lowering effects displayed by the two drugs, administered at the same dosage (1.0 gm/kg body weight) and to the same patient, are shown in Figure 7.

To date no side-effects related to the long-term usage of sodium ascorbate have been

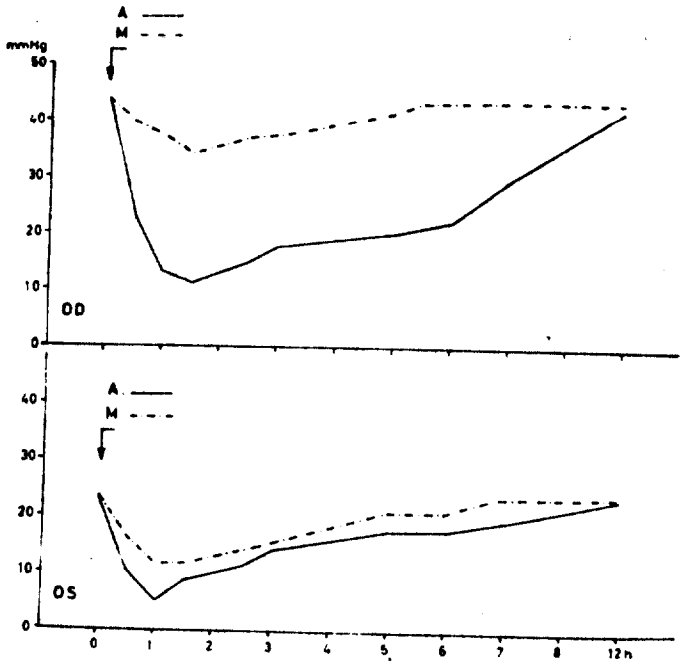


Fig. 7 (Virno, Bucci, Pecori-Giraldi and Cantore). Comparative hypotensive effect on congenital glaucoma of the right eye and on normal intraocular pressure of the left eye of sodium ascorbate and of mannitol. At arrow: administration of 1.0 gm/kg body weight intravenously of 20% sodium ascorbate (A) and, 24 hours later, administration of 1.0 gm/kg body weight of 20% mannitol (M).

noted in the 56 neurosurgical patients treated. The results of these studies will be published in the near future.

II. Sixteen patients suffering from various types of glaucoma have been treated with combined glycerol-sodium ascorbate. There were four cases of acute congestive glaucoma, one case of chronic secondary glaucoma, six cases of chronic congestive glaucoma, four cases of secondary glaucoma and one case of hemorrhagic glaucoma.

All patients received 2.0 ml/kg body weight of the glycerol-sodium ascorbate solution (0.6 gm/kg body weight of glycerol [30% solution] and 0.28 gm/kg body weight of sodium ascorbate [20% solution]) intravenously.

Tonometric readings were taken before treatment and at 10-, 20-, 30-, 60- and 120-minute intervals following administration. The maximum hypotensive effect was observed between 30 and 60 minutes from the start of the infusion. Administration time varied with the volume of the solution infused and, consequently, to the body weight. Usually, infusion lasted 15 to 20 minutes.

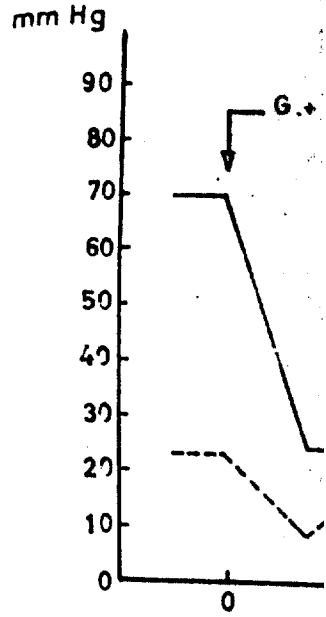
Figures 8 and 9 show the reduction in pressure obtained with intravenous administration of 0.6 gm/kg body weight glycerol combined with 0.28 gm/kg body weight sodium ascorbate in an eye with glaucoma following cataract extraction and in an eye with congestive glaucoma.

No significant blood pressure fluctuations were noted during and following the administration. No evidence of hematuria has been found in any patient. No side-effects have ever been encountered.

Conclusions

The results obtained, both in the experimental animal and in man, with the intravenous use of vitamin C (sodium salt) showed that this drug, as a 20% solution at pH 7.2 to 7.4, at the doses of 0.4 to 1.0 gm/kg body weight, may be included among the most effective osmotic agents able significantly to reduce intraocular pressure.

We are not aware of any previous work, from a laboratory or clinic, describing the osmotic action of sodium ascorbate. Preceding research work, carried out by us on the acute and chronic toxicity of this drug,[30]



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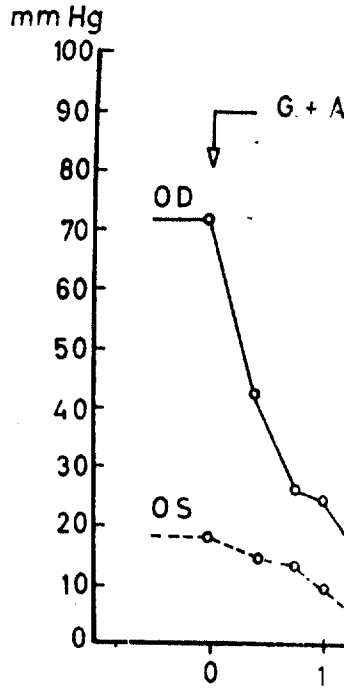

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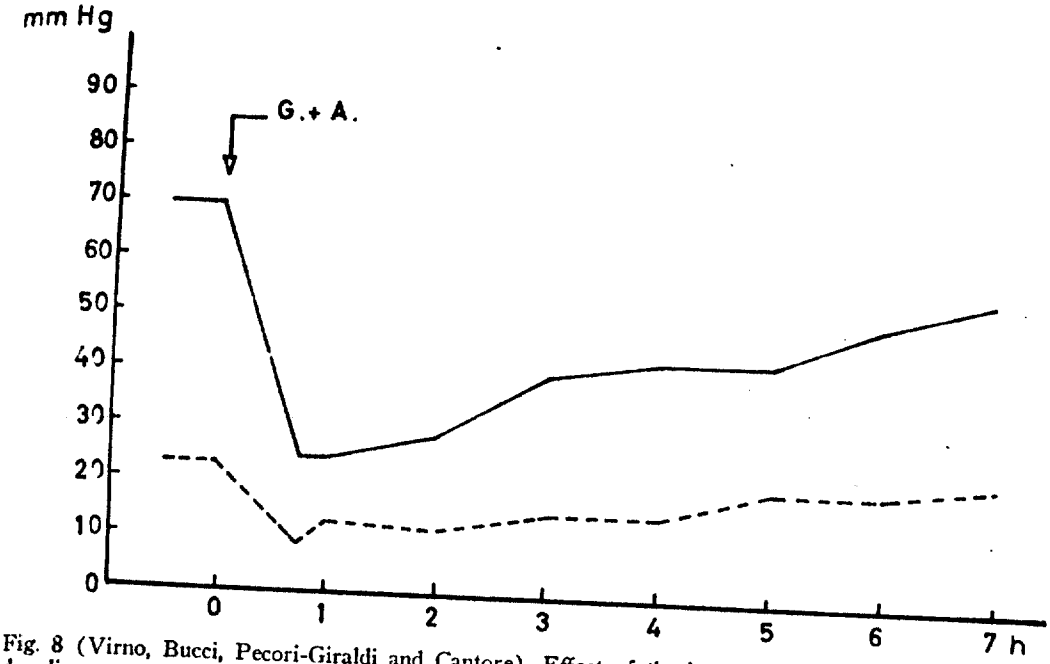

Fig. 8 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of the intravenous combination of glycerol and sodium ascorbate on secondary glaucoma following cataract extraction of the right eye. At (G+A): 0.6 gm/kg body weight of glycerol (30% solution) and 0.28 gm/kg of sodium ascorbate (20% solution) were given intravenously. Infusion time was 22 minutes. Maximum hypotensive effect was attained within 45 minutes from the start of the infusion.

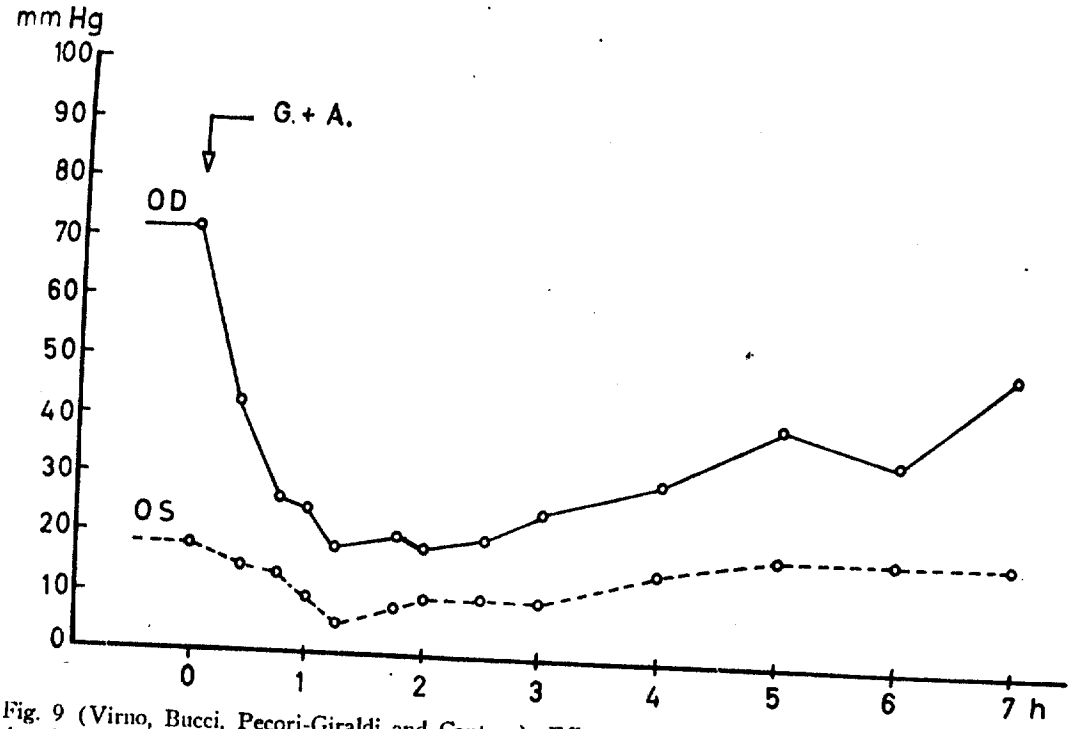

Fig. 9 (Virno, Bucci, Pecori-Giraldi and Cantore). Effect of the intravenous combination of glycerol and sodium ascorbate on congestive glaucoma of the right eye. At (G+A): 0.6 gm/kg body weight of glycerol (30% solution) and 0.28 gm/kg body weight of sodium ascorbate (20% solution) were given intravenously: Infusion time was 25 minutes.

pointed out that, at the doses of 1.5 gm/kg body weight intravenously, sodium ascorbate did not produce any untoward side-effects, provided the pH of the solution did not shift from values of 7.2 to 7.4. If the pH of the solution was higher or lower than 7.2—7.4, significant pressure fluctuations occurred in the animal.

The osmotic effect displayed by 20% sodium ascorbate was found to be truly remarkable. When compared with mannitol, sodium ascorbate was shown to be significantly more effective in lowering intraocular hypertension, at the same dosage and in the same patient (fig. 7).

In consideration of the marked hypotensive osmotic action of sodium ascorbate and of its ability to make safe and possible the intravenous administration of glycerol,[26] we investigated the effects on intraocular pressure of a combination of these two drugs, both in the experimental animal and in man.

The results showed that, by combining these two highly effective osmotic agents and by administering them by intravenous infusion, the following effects could be obtained:

1. Marked lowering of intraocular pressure with fairly low dosages of both drugs: 0.6 gm/kg body weight of glycerol and 0.28 gm/kg body weight of sodium ascorbate.

2. Complete elimination of hematuria induced by intravenous administration of glycerol.

3. Considerable reduction of solution volume as compared with other osmotic agents (about 140 ml [2 ml/kg] in a patient weighing 70 kg, instead of 600 to 800 ml of mannitol).

4. Shortening of infusion time which, at a rate of 110 drops per minute, would be of about 15 to 20 minutes (at the same rate, mannitol infusion to obtain the same hypotensive effect would last 90 minutes).

5. Maximum tension-lowering effect attained in a very short time as compared with the other osmotic agents.

6. Marked effectiveness at doses of 0.6 gm/kg of glycerol and 0.28 gm/kg of sodium ascorbate, which are far from lethal ones (intravenous lethal doses of glycerol and sodium ascorbate were found to be, respectively, 11-12 gm/kg and 6-7 gm/kg[30] in the experimental rabbit.

7. Lack of toxic side-effects.

In all our trials, no untoward complications have been encountered following the use of sodium ascorbate or of combined glycerol-sodium ascorbate. Only two patients, treated with the combined medication, experienced some degree of headache.

The combination of glycerol-sodium ascorbate has proven to be a highly effective intravenous osmotic method for the reduction of intraocular pressure.

Summary

The experimental and clinical results obtained with the intravenous use of vitamin C (sodium salt) as an agent for the osmotic reduction of intraocular pressure are presented. The method of its administration and some case reports are described. In a dosage of 0.4 to 1.0 gm/kg body weight intravenously, administered as a 20% solution at pH 7.2 to 7.4, vitamin C (sodium salt) induces marked ocular hypotony in approximately 60 to 90 minutes.

Furthermore, vitamin C (sodium salt) has proved useful in completely eliminating the hematuria produced by the intravenous administration of glycerol. No evidence of hematuria after intravenous administration of 30% glycerol combined with 20% vitamin C (sodium salt) was found.

The experimental and clinical results show that a combination of glycerol-sodium ascorbate is a highly effective intravenous osmotic method to produce a significant reduction in intraocular pressure.

Viale Regina, Elena, 289
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Clinica Oculistica dell'Universita' di Roma

Changes in osmotic pressure of the blood and in intraocular tension following intravenous infusion of strong doses of vitamin C (sodium ascorbate)

By G. B. Moschini

The use of osmotic substances in the treatment of acute endocranial and intraocular hypertension has become constant practice in recent years. Instead of magnesium sulfate, dextrose or sorbitol, the substances most commonly used are urea, glycerol and mannitol.

The possibility of using some of these substances for intravenous infusion and thus creating in a short time a strong osmotic gradient between blood and intraocular fluids with consequent rapid decrease of the intraocular tension has prompted numerous researchers to study substances of low molecular weight, non-toxic and with relatively little tendency to cross the hemato-ophthalmic barrier, in the hope of finding an ideal substance with respect to fast action, absence of acute and chronic toxicity and side effects, low cost, and possibility of preservation in the lyophilized state.

On the basis of some research done by Baur and Staub and by Kirchmair and Kirsch, which evidenced increased diuresis following therapy with vitamin C by mouth at the dose of 10 grams, in viral hepatitis, Virno et al. were the first to undertake to study the possibility of further increasing the doses of vitamin C so as to obtain a diuretic effect that could well be utilized in therapy.

After having demonstrated the perfect tolerance of vitamin C (sodium ascorbate) given intravenously in animals up to a dose of 5 g/kg and the absence of chronic toxicity (Della Rocca, Pecori-Giraldi, Cantore, Virno), Virno et al. showed that in man the dose of 1 g/kg causes a considerable increase in diuresis, which on the average is doubled.

Having ascertained that the mechanism whereby this substance acts is of an osmotic nature, Virno, Bucci, Pecori-Giraldi, Cantore carried out investigations on the relationships between sodium ascorbate and ocular tension, demonstrating that this substance infused intravenously in man at the dose of 1 g/kg is cap-

able of lowering the ocular pressure more than equal doses of other osmotic substances commonly used.

We have undertaken in the present paper to study the variations of the osmotic pressure of the blood, its relationships with the concentration of vitamin C in the blood, and the variations of the ocular tension after intravenous administration of ascorbic acid (sodium ascorbate) at doses of 0.1, 0.2, 0.5 and 1 g/kg.

Methods

We studied 18 patients varying in age between 30 and 70 years, with various forms of ocular hypertension.

We used pure, crystallized vitamin C (Merck 500074), dissolved in twice distilled water at the concentration of 20%, buffered to pH 7.4 with sodium bicarbonate.

The solutions were sterilized by means of a membrane filter G D 15 (Membran-filter Göttingen) with membrane M F/10, and they were used within 48 hours of their preparation.

The rate of the intravenous infusion was 60-80 drops per minute.

The osmotic pressure of the blood and of the urine was evaluated with the Fischer osmometer Model G.

The concentration of the ascorbic acid in the blood and in the urine was measured with the method of Sullivan and Clarke modified by Bonomi.

Results

Intravenous infusion of sodium ascorbate at the dose of 1 g/kg causes a rapid decrease of the ocular tension both in acute and in chronic simple or irritative glaucoma.

Fig. 1. Action of sodium ascorbate on ocular tension and on osmotic pressure of the blood in a case of chronic irritative glaucoma. At "A" administration of 1 g/kg body weight of 20% sodium ascorbate intravenously.

Fig. 2. Action of sodium ascorbate on ocular tension and on osmotic pressure of the blood in a case of acute glaucoma. At "A" administration of 0.5

g/kg body weight of 20% sodium ascorbate intravenously.

Fig. 3. Action of sodium ascorbate on ocular tension and on osmotic pressure of the blood in a case of acute glaucoma. At "A" administration of 0.2 g/kg body weight of 20% sodium ascorbate intravenously.

The minimum tonometric levels are reached between the first and second hour; then there is a progressive return toward the starting values. At the same time an increae of the osmotic pressure of the blood occurs, which is maximum at the end of the infusion, about 31 m osm above the initial values, and which progressively decreases returning to the starting levels within 6-12 hours.

The ascorbinemia curve matches that of the osmotic pressure of the blood: Mean maximum level of 495 mg% at the end of the intravenous infusion, progressive return to normal within 6-8 hours. In the same period there is an increase of the diuresis, and the concentration of vitamin C in the urine varies, according to the patients, from a minimum of 3 g% to a maximum of 7 g%.

At the dose of 0.5 g/kg the mean increase of the osmotic pressure of the blood at the end of the infusion is identical with the mean value found after a dose of 1 g/kg; the return to the starting values occurs within 6 hours. The urinary excretion of vitamin C is highest during the first 2 hours, 3-5 g%; toward the sixth hour only minimal traces are detectable.

Traces

As to the ocular tension, we have a maximum reduction between the first and second hour and a progressive return toward the initial values, which are reached between the sixth and the twelfth hour. The greatest pressure-reducing effect was noted in the cases of acute glaucoma.

The mean value of the ascorbinemia at the end of the infusion is 480 mg%, after 1 hour 400 mg%, and after 4 hours 70 mg%.

At a dose of 0.2 g/kg the pressure-reducing action is somewhat less than that obtained with higher doses of sodium ascorbate. However, there are reductions of about 20-25 mm Hg with respect to the initial values. The osmotic pressure of the blood increases by about 15 m osm and returns to normal in the fourth hour; the vitamin C concentration in the blood reaches an initial maxi-

mum of about 252 mg%; in the fourth hour a progressive return to the starting values occurs.

At a dose of 0.1 g/kg there is an average reduction of the ocular tension of 11 mm Hg; the increase of the osmotic pressure and of the vitamin C concentration in the blood at the end of the infusion are 5 m osm and 75 mg%, respectively.

Conclusions

It is clear from the analysis of the results of our experiments that an increase of the osmotic pressure of the blood, following intravenous infusion of sodium ascorbate is always accompanied by a reduction of the ocular tension, which reduction is not in a fixed and constant ratio with the increase of the osmotic pressure, but varies according to the subjects and the type of glaucoma.

It is further noted that the individual variations of the osmotic pressure after the same dose of sodium ascorbate are sometimes wide, various factors (renal function, hydric state, etc.) being involved in the mechanism of action of the osmotic substances.

In some patients, in fact, we have observed at the end of the intravenous infusion a prompt and conspicuous urinary excretion of vitamin C, 4-7%, indicative of a high capacity of concentration by the kidney, accompanied by a relatively small increase of the osmotic pressure of the blood and a pressure-reducing action on the ocular tension smaller than that noted in other subjects in whom the urinary excretion of the vitamin C did not go beyond a maximum concentration of 3-4 g% and in which the osmotic pressure of the blood increased to 40-47 m osm.

We believe we can rule out, on the basis of theoretical considerations and on the basis of a careful evaluation of the data listed, that the sodium bicarbonate used to buffer the acid pH of vitamin C is involved even in small measure in causing the increase of the osmotic pressure of the blood; in fact, taking into account the ascorbinemic concentration, the variations of the osmotic pressure, the molecular weight of ascorbic acid and its non-dissociability in aqueous solution, there appears to be between the values found by us a relation-

ship almost identical with that theoretically predictable between a given concentration of vitamin C in the blood and the respective increase of the osmotic pressure.

In view of the results obtained, we can affirm that the dose of 0.5 g/kg of vitamin C (sodium ascorbate) has proved efficient in most cases in significantly reducing the ocular tension. We do not think it desirable to increase this dose since further increase of the osmotic pressure has not been observed and on the other hand the administration of a greater dose may lead to a great need to introduce liquids, so that the action of the product would in part be neutralized.

Summary (see p. 151 of Italian publication)

Table I

A = Tension in mm Hg

B = Osmotic pressure of blood in m osm

C = Ascorinemia in mg%

Type of glaucoma	Time	O	20 min	40 min	60 min	2h	4h	6h	12h
Irritative chronic	A	O D	38	35	35	37	19	27	
Vitamin Cl g/kg		O S	55	47	33	25	28	39	
	B		310	342		348	323		
	C		1.5	525		590	210		
Irritative chronic	A	O D	44	35	22		18	26	34
Marchesani's syndrome		O S	60	42	30		27	33	48
Vitamin C 1 g/kg	B		307	331		325		318	309
Chronic simple	A	O D	36	29	24	15	19		32
Vitamin C 1 g/kg		O S	28	25	21	21	23		25
	B		316	346		340	325		312
	C		1	585		460	200		
Acute	A	O D	65	38	32	25	36		54
Vitamin C 1 g/kg	B		305	340		340	314		
	C		1.5	515		465	160		
Acute	A	O D	50	47	40	31	27		44
Vitamin C 0.5 g/kg	B		302	344		335	310		
	C		1.5	680		500	150		
Acute	A	O D	65	57	45	30	35	42	
Vitamin C 0.5 g/kg	B		312	359		344		315	
	C		1.5	535		480		20	

Type of glaucoma	Time	0	20 min	40 min	60 min	2 h	4 h	6 h	12 h
Acute	A O D	48	41		26	28		24	
Vitamin C 0.5 g/kg	B	295	323		315		308		
	C	1.5	435		340		160		
Acute	A O D	57	46	35	23	30		38	
Vitamin C 0.5 g/kg	B	321	346		344		327		
	C	1	415		340		60		
Chronic simple	A O D	24	18		15				20
Vitamin C 0.5 g/kg	O S	31	26		20				25
	B	323	348		332				25
Chronic simple	A O D	37	30		20			24	18
Vitamin C 0.5 g/kg	O S	30	26		19			22	26
	B	303	325		327			305	
	C	1.5	465		418			125	
Acute	A O D	40	36	27	25	30		35	
Vitamin C 0.2 g/kg	B	310	325		322		313		
	C	1.5	260		185		60		
Acute	A O D	54	47	44	31	28	37		
Vitamin C 0.2 g/kg	B	328	346		341		330		
	C	1	285		200		25		
Chronic simple	A O D	40	40	36	33	37	42		
Vitamin C 0.2 g/kg	O S	32	30	25	27	23	27		
	B	315	327		322		314		
	C	1.5	215		160		10		
Hypertensive uveitis	A O S	48	36	32	23	25	22	26	
Vitamin C 0.2 g/kg	B	311	327		324		315		
	C	1.5	250		215		30		
Chronic simple	A O D	35	33		24		26		37
Vitamin C 0.1 g/kg	O S	38	33		29		27		34
	B	302	307		305		301		
	C	1.5	80		58				
Chronic simple	A O D	42	42		31	36			39
	O S	29	25		23	19			308
	B	309	313		310				
	C	1.5	65		20				
Acute	A O S	55	50	47	40	35		48	
Vitamin C 0.1 g/kg	B	297	304		300				
	C	1	115		34				
Irritative chronic	A O S	38	36	35	32	40			
Vitamin C 0.1 g/mg	B	312	315		315				
	C	1.5	40		10				

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Direttore: Prof. G. B. BIETTI

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MODIFICAZIONI DELLA PRESSIONE OSMOTICA EMATICA E DEL TONO OCULARE A SEGUITO DI INFUSIONE ENDOVENOSA DI FORTI DOSI DI VITAMINA C (ASCORBATO DI SODIO)

G. B. MOSCHINI

L'uso di sostanze osmotiche nel trattamento della ipertensione endocranica ed endoculare acuta è divenuto in questi ultimi anni una pratica costante. Abbandonato ormai il solfato di magnesio, il destrosio, il sorbitolo, le sostanze piú comunemente usate sono l'urea, il glicerolo e il mannitolo.

La possibilità di usare alcune di queste sostanze per infusione endovenosa e creare quindi in breve tempo un forte gradiente osmotico tra sangue e fluidi intraoculari con conseguente rapida caduta del tono oculare ha spinto numerosi ricercatori allo studio di sostanze a piccolo peso molecolare, non tossiche e con relativamente scarsa tendenza ad attraversare la barriera emato-oftalmica, nella speranza di trovare una sostanza ideale per rapidità d'azione, assenza di tossicità acuta e cronica e di effetti collaterali, basso costo, possibilità di conservazione eventualmente allo stato liofilizzato.

Partendo da alcune ricerche condotte da BAUR e STAUB e da KIRCHMAIR e KIRSCH, in cui veniva messo in evidenza l'aumento della diuresi a seguito di terapia con vitamina C per os: alla dose di 10 grammi, nella epatite virale, VIRNO e Coll. per primi, si proposero di studiare la possibilità di aumentare ulteriormente le dosi di vitamina C in modo da ottenere un effetto diuretico tale da essere utilmente sfruttato in terapia.

Dopo aver dimostrato la perfetta tollerabilità della vitamina C (ascorbato di sodio) somministrata endovena nell'animale fino alla dose di 5 g. pro/Kg. e l'assenza di tossicità cronica (DELLA ROCCA, PECORI-GIRALDI, CANTORE, VIRNO), è stato da VIRNO e

Coll. messo in evidenza come nell'uomo la dose di 1 g. pro/Kg. provochi un notevole aumento della diuresi che viene in media raddoppiata.

Una volta accertato che il meccanismo con cui agisce questa sostanza è di tipo osmotico, VIRNO, BUCCI, PECORI-GIRALDI, CANTORE hanno compiuto indagini sui rapporti tra ascorbato di sodio e tono oculare, mettendo in evidenza come questa sostanza infusa endovena nell'uomo alla dose di 1 g. pro/Kg. sia capace di abbassare la pressione oculare assai più che non eguali dosi delle altre sostanze osmotiche comunemente usate.

Nel presente lavoro ci siamo proposti di studiare le variazioni della pressione osmotica del sangue, i suoi rapporti con la concentrazione ematica della vitamina C, le variazioni del tono oculare dopo somministrazione endovenosa di acido ascorbico (ascorbato di sodio) alla dose di 0.1, 0.2, 0.5, 1 g. pro/Kg.

METODI

Sono stati studiati n. 18 pazienti di età variante dai 30 ai 70 anni, affetti da varie forme di ipertono oculare.

Si è usata vitamina C pura, cristallizzata (Merck 500074), sciolta in acqua bidistillata alla concentrazione del 20%, tamponata a pH 7.4 con bicarbonato di sodio.

Le soluzioni venivano sterilizzate mediante filtro a membrana G D 15 (Membranfilter Göttingen) con membrana M F/10 e venivano usate entro 48 ore dalla preparazione.

La velocità della infusione endovenosa era di 60-80 gocce al minuto.

La pressione osmotica del sangue e delle urine è stata valutata mediante l'osmometro di Fische mod. G.

La concentrazione dell'acido ascorbico nel sangue e nelle urine è stata misurata con il metodo di Sullivan e Clarke modificato da BONOMI.

RISULTATI

L'infusione endovenosa di ascorbato di sodio alla dose di 1 g. pro/Kg. provoca una rapida caduta del tono oculare sia nel glaucoma acuto che nel glaucoma cronico semplice od irritativo.

I livelli tonometrici minimi vengono raggiunti tra la prima

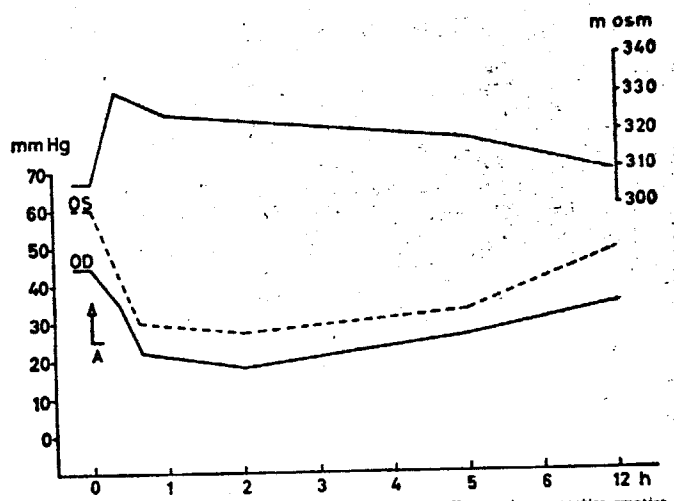

Fig. 1. - Azione dell'Ascorbato di sodio sul tono oculare e sulla pressione osmotica ematica in un caso di glaucoma cronico irritativo. In « A » somministrazione di 1 g/Kg di peso corporeo di Ascorbato di sodio al 20% per via endovenosa.

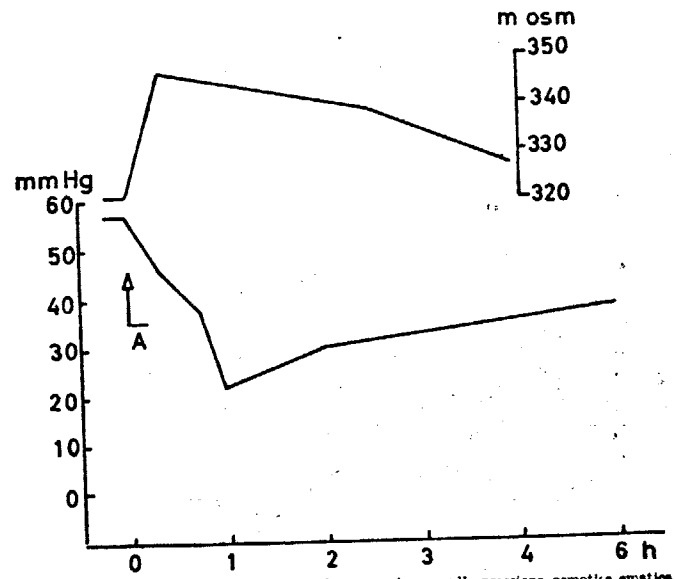

Fig. 2. - Azione dell'Ascorbato di sodio sul tono oculare e sulla pressione osmotica ematica in un caso di glaucoma acuto. In « A » somministrazione di 0.5 g/Kg di peso corporeo di Ascorbato di sodio al 20% per via endovenosa.

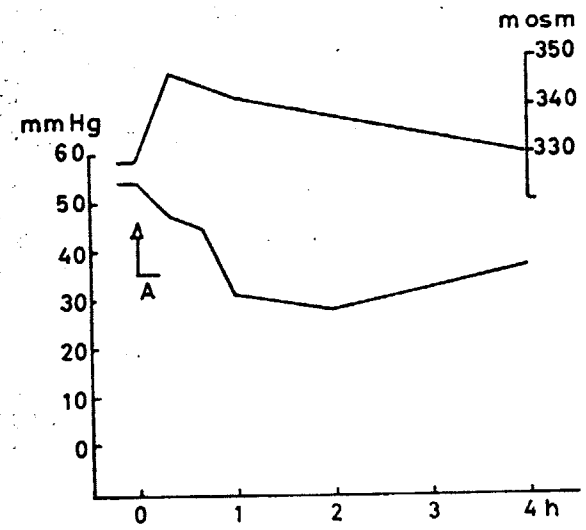

Fig. 3. - Azione dell'Ascorbato di sodio sul tono oculare e sulla pressione osmotica ematica in un caso di glaucoma acuto. In « A » somministrazione di 0.2 g/Kg di peso corporeo di Ascorbato di sodio al 20% per via endovenosa.

e la seconda ora; in seguito si ha un progressivo ritorno verso i valori di partenza. Contemporaneamente si assiste ad un aumento della pressione osmotica del sangue, aumento che è massimo al termine della infusione, circa 31 m osm al di sopra dei valori iniziali e che progressivamente si riduce tornando ai livelli di partenza nello spazio di 6-12 ore.

L'andamento dell'ascorbinemia è sovrapponibile a quello della pressione osmotica ematica: livello massimo medio di 495 mg% alla fine della infusione endovenosa, progressivo ritorno alla norma entro 6-8 ore. Nello stesso periodo si ha un aumento della diuresi e la concentrazione della vitamina C nelle urine varia, a seconda dei pazienti, da un minimo di 3 g% ad un massimo di 7 g%.

Con la dose di 0.5 g. pro/Kg. l'aumento medio della pressione osmotica del sangue al termine della infusione è identico al valore medio trovato dopo una dose di 1 g. pro/Kg.; il ritorno ai valori di partenza si ha entro 6 ore. L'escrezione urinaria di vitamina C è massima nelle prime 2 ore, 3-5 g%; verso la sesta ora sono dosabili solo tracce minime.

Per quanto concerne il tono oculare, si ha una diminuzione massima tra la prima e la seconda ora ed un progressivo ritorno verso i valori iniziali che vengono raggiunti tra la sesta e la dodicesima ora. Il massimo effetto ipotonizzante è stato notato nei casi di glaucoma acuto.

Il valore medio dell'ascorbinemia al termine della infusione è di 480 mg%, dopo 1 ora di 400 mg%, dopo 4 ore di 70 mg%.

Con una dose di 0.2 g pro/Kg. l'azione ipotonizzante è alquanto inferiore rispetto a quella ottenuta con dosi maggiori di ascorbato di sodio, tuttavia si hanno diminuzioni di circa 20-25 mm Hg rispetto ai valori iniziali; la pressione osmotica del sangue aumenta di circa 15 m osm e torna alla norma entro la quarta ora, la concentrazione ematica della vitamina C raggiunge un valore massimo iniziale di circa 252 mg%, entro la quarta ora si ha un ritorno progressivo ai valori di partenza.

Con una dose di 0.1 g. pro/Kg. si ha una diminuzione media del tono oculare di 11 mm Hg; l'aumento della pressione osmotica e della concentrazione ematica della vitamina C al termine della infusione sono rispettivamente di 5 m osm e di 75 mg%.

Conclusioni

Dall'analisi dei risultati delle esperienze da noi condotte, risulta chiaro che ad un aumento della pressione osmotica ematica, in seguito ad infusione endovenosa di ascorbato di sodio, corrisponde sempre una diminuzione del tono oculare, diminuzione che non è in un rapporto fisso e costante con l'incremento della pressione osmotica, ma variante a seconda dei soggetti e del tipo di glaucoma.

Va inoltre notato che le variazioni individuali della pressione osmotica dopo una stessa dose di ascorbato di sodio sono talora ampie, intervenendo nel meccanismo d'azione delle sostanze osmotiche vari fattori (funzionalità renale, stato idrico, ecc.).

Si è osservato infatti in taluni pazienti al termine della infusione endovenosa una pronta e cospicua escrezione urinaria di vitamina C, 4-7 grammi %, indice questo di una elevata capacità di concentrazione da parte del rene, accompagnata da un

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TABELLA I.

Glaucoma di tipo:	tempo 0		20'	40'	60'	2h	4h	6h	12h
Irritativo cronico Vitamina C 1 g/Kg	A Tono in mm. Hg O.D.	38	35	27	19	20		27	
	Tono in mm. Hg O.S.	55	47	33	25	28		39	
	B Pressione osmotica del sangue in m osm.	310	342		348		323		
	C Ascorbinemia in mg%	1.5	525		590		210		
Irritativo cronico Sindrome di Marchesani Vitamina C 1 g/Kg	Tono in mm. Hg O.D.	44	35	22		18		26	34
	Tono in mm. Hg O.S.	60	42	30		27		33	48
	Pres. osm. m osm.....	307	331		325			318	309
	Ascorbinemia in mg%.....	1.5	350		380			180	20
Cronico semplice Vitamina C 1 g/Kg	Tono in mm. Hg O.D.	36	29	24	15		19		32
	Tono in mm. Hg O.S.	28	25	21	21		23		25
	Pres. osm. m osm.....	316	346		340		325		312
	Ascorbinemia in mg%.....	1	585		460		200		
Acuto Vitamina C 1 g/Kg	Tono in mm. Hg O.D.	65	38	32	25		36		54
	Pres. osm. m osm.....	305	340		340			314	
	Ascorbinemia in mg%.....	1.5	515		465			160	
Acuto Vitamina C 0.5 g/Kg	Tono in mm. Hg O.D.	50	47	40	31	27			44
	Pres. osm. m osm.....	302	344		335		310		
	Ascorbinemia in mg%.....	1.5	680		500		150		
Acuto Vitamina C 0.5 g/Kg	Tono in mm. Hg O.S.	65	57	45	30	35		42	
	Pres. osm. m osm.....	312	359		344			315	
	Ascorbinemia in mg%.....	1.5	535		480			20	
Acuto Vitamina C 0.5 g/Kg	Tono in mm. Hg O.D.	48	41		26	28		24	
	Pres. osm. m osm.....	295	323		315		308		
	Ascorbinemia in mg%.....	1.5	435		340		160		
Acuto Vitamina C 0.5 g/Kg	Tono in mm. Hg O.D.	57	46	35	23	30		38	
	Pres. osm. m osm.....	321	346		344		327		
	Ascorbinemia in mg%.....	1	415		340		60		
Cronico semplice Vitamina C 0.5 g/Kg	Tono in mm. Hg O.D.	24	18		15				20
	Tono in mm. Hg O.S.	31	26		20				25
	Pres. osm. m osm.....	323	348		332				

Glaucoma di tipo:	tempo 0		20'	40'	60'	2h	4h	6h	12h
Cronico semplice Vitamina C 0.5 g/Kg	Tono in mm. Hg O.D.	37	30		20			24	18
	Tono in mm. Hg O.S.	30	26		19			22	26
	Pres. osm. m osm.....	303	325		327			305	
	Ascorbinemia in mg%.....	1.5	465		418			125	
Acuto Vitamina C 0.2 g/Kg	Tono in mm. Hg O.D.	40	36	27	25	30		35	
	Pres. osm. m osm.....	310	325		322		313		
	Ascorbinemia in mg%.....	1.5	260		185		60		
Acuto Vitamina C 0.2 g/Kg	Tono in mm. Hg O.D.	54	47	44	31	28	37		
	Pres. osm. m osm.....	328	346		341		330		
	Ascorbinemia in mg%.....	1	285		200		25		
Cronico semplice Vitamina C 0.2 g/Kg	Tono in mm. Hg O.D.	40	40	36	33	37	42		
	Tono in mm. Hg O.S.	32	30	25	27	23	27		
	Pres. osm. m osm.....	315	327		322		314		
	Ascorbinemia in mg%.....	1.5	215		160		10		
Uveite ipertensiva Vitamina C 0.2 g/Kg	Tono in mm. Hg O.S.	48	36	32	23	25	22	26	
	Pres. osm. m osm.....	311	327		324		315		
	Ascorbinemia in mg%.....	1.5	250		215		30		
Cronico semplice Vitamina C 0.1 g/Kg	Tono in mm. Hg O.D.	35	33		24		26		37
	Tono in mm. Hg O.S.	38	33		29		27		34
	Pres. osm. m osm.....	302	307		305		301		
	Ascorbinemia in mg%.....	1.5	80		58				
Cronico semplice Vitamina C 0.1 g/Kg	Tono in mm. Hg O.D.	42	42		31	36			39
	Tono in mm. Hg O.S.	29	25		23	19			27
	Pres. osm. m osm.....	309	313		310				308
	Ascorbinemia in mg%.....	1.5	65		20				
Acuto Vitamina C 0.1 g/Kg	Tono in mm. Hg O.S.	55	50	47	40	35		48	
	Pres. osm. m osm.....	297	304		300				
	Ascorbinemia in mg%.....	1	115		34				
Irritativo cronico Vitamina C 0.1 g/mg	Tono in mm. Hg O.S.	38	36	35	32	40			
	Pres. osm. m osm.....	312	315		315				
	Ascorbinemia in mg%.....	1.5	40		10				

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relativamente scarso incremento della pressione osmotica ematica ed una azione ipotonizzante sul tono oculare inferiore a quella notata in altri soggetti nei quali l'escrezione urinaria della vitamina C non andava oltre una concentrazione massima di 3-4 grammi % e nei quali la pressione osmotica ematica aumentava sino a 40-47 m osm.

Riteniamo che sia da escludere in base a considerazioni teoriche ed in base ad una attenta valutazione dei dati elencati, che il bicarbonato di sodio, utilizzato per tamponare il pH acido della vitamina C, intervenga sia pure in lieve misura nel determinismo dell'incremento della pressione osmotica ematica; infatti, tenendo presente il tasso ascorbinemico, le variazioni della pressione osmotica, il peso molecolare dell'acido ascorbico e la sua non dissociabilità in soluzione acquosa, risulta esservi tra i valori da noi trovati un rapporto pressoché identico a quello teoricamente prevedibile tra una data concentrazione ematica di vitamina C ed il corrispettivo incremento della pressione osmotica.

In considerazione dei risultati ottenuti possiamo affermare che la dose di 0.5 g. pro/Kg. di vitamina C (ascorbato di sodio) si è rivelata efficace nella maggioranza dei casi nel ridurre in maniera significativa la tensione oculare. Non riteniamo opportuno aumentare tale dóse in quanto non è stato osservato un ulteriore aumento della pressione osmotica e d'altra parte la somministrazione di una dose maggiore può portare come conseguenza ad una forte necessità di introdurre liquidi, venendo in tal modo in parte neutralizzata l'azione del prodotto.

RIASSUNTO

Sono stati studiati 18 pazienti affetti da varie forme di ipertono oculare ed è stato rilevato come la somministrazione endovenosa nell'uomo del sale sodico della vitamina C alle dosi di 0.1, 0.2, 0.5, 1 g. pro/Kg. provoca un aumento della pressione osmotica ematica che varia a seconda del paziente, del tipo di glaucoma e della dose impiegata.

Contemporaneamente si nota una diminuzione del tono oculare. Vengono esaminati i rapporti tra pressione osmotica ematica, pressione endoculare e concentrazione della vitamina C nel sangue e nelle urine.

RÉSUMÉ

Moschini G. B. - *Modifications de la pression osmotique hématique et de la tension oculaire après infusion endoveineuse de hautes doses de Vitamine C (ascorbat de sodium).*

On a examiné 18 malades atteints de différents types de glaucome. On a mis en évidence que l'administration intraveineuse chez l'homme du sel sodique de la vit. C à la dose de 0.1, 0.2, 0.5, 1 g/Kg. provoque une augmentation de la pression osmotique hématique, qui varie suivant le malade, le type de glaucome et la dose employée.

On note simultanément une diminution de la tension oculaire.

On examine les rapports entre pression osmotique hématique, pression endoculaire et concentration de la vitamine C dans le sang et dans les urines.

SUMMARY

Moschini G. B. - *Modifications of the hematic osmotic pressure and of the ocular tension after intravenous infusion of high doses of CVitamin (sodium ascorbate).*

Eighteen patients affected by different types of glaucoma have been examined. It has been found that the intravenous administration in the man of the sodic acid of the C vitamin at the dosage of 0.1, 0.2, 0.5, 1 g/Kg. provokes an increase of the hematic osmotic pressure, which varies according to the patient, the type of glaucoma and the employed dose.

Simultaneously a decrease of the ocular tension is observed.

The relationships between hematic osmotic pressure, intraocular pressure and concentration of the C vitamin in the blood and the urines are examined.

ZUSAMMENFASSUNG

Moschini G. B. - *Aenderungen des hämatischen osmotischen Druckes und des Augendruckes nach intravenöser Infusion von hohen Dosen von Vitamin C (Natriumaskorbat).*

18 Patienten mit verschiedenen Formen von Glaukom wurden untersucht. Es wurde beobachtet, dass die intravenöse Verabreichung beim Menschen von dem Natriumsalz des Vitamins C, in Dosen von 0.1, 0.2, 0.5, 1 g/Kg, eine Zunahme des hämatischen osmotischen Druckes hervorruft, die je nach dem Patienten, dem Typus von Glaukom und der angewandten Dosierung verschieden ist.

Gleichzeitig beobachtet man eine Abnahme des Augendruckes.

Man nimmt die Beziehungen zwischen hämatischem osmotischem Drucke, intraokulären Drucke und Konzentration des Vitamin C im Blute und im Harne in Betracht.

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NOTIZIE

VIII CONGRESSO PANAMERICANO DI OFTALMOLOGIA

L'VIII Congresso Panamericano di Oftalmolgia avrà luogo in Mar del Plata dal 24 al 30 marzo del 1968.

Durante il Congresso si svolgerà un intenso programma; simposi, temi liberi, films, mostre scientifiche e commerciali.

Il tema ufficiale del Congresso sarà: « Trattamento delle complicazioni della chirurgia del bulbo oculare ». A questo fine, si sono organizzati i seguenti simposi:

Trattamento delle complicazioni della chirurgia della cornea - Moderatore: Raul Rodriguez Barrios (Montevideo - Uruguay).

Trattamento delle complicazioni della chirurgia della cataratta - Moderatore: Dr. Frank W. Newell (Chicago - USA)

Trattamento delle complicazioni della chirurgia del glaucoma - Moderatore: Dr. Hilton Rocha (Belo Horizonte - Brasil).

Trattamento delle complicanze della chirurgia del distacco di retina - Moderatore: Dr. Edward Norton (Miami - USA).

Sono stati nominati 12 oratori per ciascun tema.

I lavori a tema libero saranno limitati e rigorosamente selezionati. La discussione in ogni caso sarà aperta da un oftalmologo appositamente eletto.

Durante il Congresso avrà luogo il III Festival Internazionale del Film Oftalmologico.

Il Dr. Jules François è stato invitato di onore.

IL PROF. J. CHARAMIS ELETTO ALL'ACCADEMIA D'ATENE

Il Presidente della Società Ellenica di Oftalmologia ci ha annunciato l'elezione del Prof. Jean Charamis, Presidente del Consiglio Internazionale di Oftalmologia, all'Accademia di Atene.

PREMIO DELLA « SOCIETÈ D'OPHTALMOLOGIE DE L'OUEST DE LA FRANCE »

A seguito di un lascito fatto alla « Societé d'Ophtalmologie de l'Ouest de la France » un premio di 5000 franchi sarà assegnato ogni tre anni all'autore di un lavoro inedito in lingua francese sul distacco di retina, e suscettibile, secondo il desiderio del donatore, di migliorarne la prognosi.

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Retinal arterial pressure in normal subjects after oral administration of glycerol and intravenous injection of sodium ascorbate

By R. Neuschüler and A. Missiroli

It is a well-known fact that ophthalmodynamometry, measuring the retinal arterial pressure (RAP), gives exact information on the hemodynamic conditions prevailing inthe internal carotids. These vessels, carrhing a major flow of blood to the bain, cause the latter to be more or less sensitive to variations in the circulation of that region, induced by a variety of causes.

Leaving aside the behavior of the RAP when the lumen of the internal carotids is reduced, it is worth noting that the literature is rich in reports on studies made on the behavior of the RAP under various physio-pathological conditions or after intake of drugs and toxic substances. Even though the results that have been published are not always acceptable on the basis of the latest techniques of measurement and evaluation of the RAP, the determination of the latter is still of substantial informative value regarding the cephalic circulation.

A critical examination of the experiments performed shows, in fact, that the results of many studies no longer have the value that had been attributed to them, mainly because of the failure to take into proper account the evaluation of the ratio between the pressure recorded at the central artery of the retina and at the humerus. Bietti already brought out this problem in a paper on the RAP under the action of adrenalin.

From the literature, and especially the older, it appears that at one time attempts were made to condition the RAP not only by the general or regional arterial pressure (as for example precisely that existing in one carotid with respect to the other); but also by the intracranial pressure (Venco for instance found, with Baillart's method, a constant correspondence between RAP and the various states of intracranial tension), and also that increasing the ocular tension might be followed by an increase of the RAP of a reflex and

compensatory origin (Venco 1932, 1945).

The recent interpretation of ophthalmodynamometry hasnow to some extent done justice to these affirmations, by admitting not only that the arterial pressue values taken at the optic papilla measure in reality, not the pressure of the retinal central artery, but that existing in the opthalmic artery near the point of its emergence from the internal carotid, but also that the absolute retinal pressure values are of no importance, but only their ratio to the H.A.P., and it is precisely from the behavior of this ratio that a certain evaluation of the cerebral circulation can be derived (Leonardi).

That detracts to some extent from the value of a possible influence of the endocranial and presumably intraocular pressure.

In reference, however, to a recent contribution (Rubichi 1966) reporting on studies on the effects of glycerol on the RAP, noting a reduction of the values after ingestion of that drug, we wanted to study the behavior of the retinal and arm pressure values after administration of glycerol as well as of sodium ascorbate. These are, in fact, substances which act on the endocranial, ocular, and general pressure, reducing the values thereof in various degree and are therefore especially suitable for studying if ophthalmodynamometric examination could be sensitive to these variations, thus supplying information on any changes in the cerebral circulation that may have been induced.

The use of these osmotically active substances has, in fact, awakened special interest in neuro-surgery as well as in oculistics, because of the favorable results obtained in the treatment of endocranial and intraocular hypertension (Virno et al. 1963-65-66). Let it be remembered in this connection that already in 1936 Martinelli and Paolazzi studied the behaviour of the RAP after intravenous administration of glucosated hypertonic solutions.

These authors found a reduction of the RAP values, to which corresponded a reduction of the pressure of the cephalorachidian fluid, measured simultaneously.

The RAP, therefore, must be influenced by osmotic substances introduced either intravenously (glucosated hypertonic solutions) or orally (glycerol).

Now that an osmotic substance introducible intravenously, sodium ascorbate, is being experimented which is much more active than glycerol (at equal dose its activity is 5 times higher), we wanted to examine the behavior of the RAP after administration of that substance, also in relation to the results obtained with the use of glycerol.

Material and methods

Twelve normal subjects were examined after administration of glycerol at 50% at a dose of 1 g per kg of body weight, adopting the following method.

The RAP was measured by Weigelin's technique. This technique, as is known, consists in measuring the diastolic and systolic pressure, at the retina as well as at the arm, from which are derived the mean arterial pressures according to the indications of Wezler and Bögers; the values obtained are compared with those found by Weigelin in normal subjects and compiled in tables so as to establish the deviations from the norm in the case being examined.

Then 50% glycerol was administered by mouth, and after 1 hour the values of RAP and HAP were taken again. The period of 1 hour was chosen in view of the fact that the action of the substance induced by the experiment is greatest at that time, both in the brain and in the eye.

The RAP measurements were single, not repeated, so as to avoid one of the error sources of the method resulting from the sharp drop in ocular tension after repeated compressions of the bulb. If any doubtful data were encountered, the examination was repeated in another session.

The experimentation with Na ascorbate was carried out on 13 subjects, the substance being introduced in a 20% solution, in a dose of ½ g per kg of body weight, by means of phleboclysis. Also in this case, the RAP was measured with the same technique before and 1 hour after the end of the phleboclysis, under the same conditions as in the previous experimentation.

An examination of the case material shows that the dynamometric response after administration of the aforesaid substances shows a certain reduction of the RAP values compared with the base values (Tables I and II).

We shall analyze first the results obtained after ingestion of glycerol (Table

1). The values we report refer to the maximum and minimum pressure as well as the mean of both, obtained by the formula of Wezler and Böger (minimum arterial pressure to which is added 42% of the differential).

The tests were made for each eye; the differences between the two parts were found to be negligible, for the mean value as well as for the maximum and minimum RAP separately.

As can be seen, in three cases there was practically no variation in the RAP values after glycerol (we refer to the mean values) (Nos. 6, 7, 10); in five cases there was a variation from 3 to 6 Hg (Nos. 1, 2, 5, 9, 11), and in three cases, greater than 6 up to 13 mm Hg (Nos. 3, 4, 12). Taking the maximum and minimum levels separately, the reduction of the RAP values, when one was found, was percentually similar for the two measurements, if somewhat lower for the maximum RAP. Another datum is taken into account at this point, namely the behavior of the HAP, which showed a tendency to a modest reduction of the value in six of the cases examined, where also the RAP was reduced in varying degree. However, it has also be found unchanged precisely where the most marked reductions of the RAP had been found (case No. 4). The reduction was more sensitive with respect to its maximum values and varied between 4 and 20 mm Hg, considering the mean value. With respect to the ocular tension, as was to be expected, and as has now been confirmed by extensive experimentation, its values were reduced after glycerol in almost equal amount in both eyes, by a minimum of 4 to a maximum of 9 mm Hg.

We shall now report the values in Table 2 which refer to subjects to whom a charge of Na ascorbate had been applied.

Also in these 13 cases, a certain tendency to reduction of the RAP values was observed, without a significant difference between the two sides. No. 8 proved scarcely sensitive to the action of sodium ascorbate, while in the other cases there appeared downward variations of the RAP values, by from 3 to 6 mm Hg (Nos. 1, 3, 7, 11) and as much as 13 mm Hg (No. 9). These values refer to the mean RAP, and if we analyze separately the maximum and minimum RAP, we see, as was observed before for the cases treated with glycerol, that the reduction

of the two values is percentually similar; with a prevalent reduction for the maximum values. The HAP remained unchanged in five cases, in one of which (No. 5) was found a corresponding evident reduction of the RAP; in the other cases it was reduced by 5 to 10 mm Hg for the mean value, the maximum more so than the minimum.

As in the cases treated with glycerol, the intraocular tension showed a significant reduction (by 4 to 9 mm Hg), equal in both eyes.

At this point, however, the examination of the data obtained by means of Weigelin's tables shows that although the VAP value, considered separately, evidences a more or less indicative reduction after intake of the osmotic substances used the retinal-humeral ratio, the only parameter from which an evaluation of the intracranial blood circulation can be drawn, remains generally within the limits of the norm indicated by the method (+0 -6 mm Hg difference from the theoretical value indicated by the tables), as can be seen from Tables 3 and 4.

Considerations and conclusions

As we said in the introduction, it has been the purpose of our research to study the ophthalmodynamometric changes that may be induced by two osmotically active substances - glycerol and Na ascorbate. So, with the presuppositions which have demonstrated clinically and experimentally as an undeniable datum the great power of dehydration and hence reduction of the cerebral mass and of the endocranial pressure, as well as of the intraocular tension, and also of the general pressure, brought about by the two substances used, and examining separately the absolute values of the RAP, we might erroneously be led to believe in a vasodilatation of the cephalic region consequent to reduced tension of the dehydrated cerebral mass, in which, therefore, intracranial circulation would be facilitated.

In reality this is not so. In fact, the examination of the retinal-humeral ratio - which, as we have said, is the only way to get to a real evaluation of the cephalic circulation according to Weigelin's technique - shows no disequilibrium between the generaland the retinal pressure values. On the contrary,

the theoretical and the actual values are in general found to match. In this connection we wish to add that also the statistical evaluation of the data obtained after glycerol and after Na ascorbate brought out no significance of the results. All this may mean that if there has been a modification of the cerebral circulation, either it was well compensated by the general circulation, or the modifications of the various parameters necessary for the evaluation of the RAP that may have been induced demonstrated a uniformity of behavior under the action of the tested substances. We refer in particular to the variations in ocular tension which, as can be seen from the case material and as is now known, are very significant. Indeed we have often found ocular tensions which under the action of the substances used showed values much below the minima appearing in the tables used for the calculation of the RAP.

In the formulation of the calculations necessary for obtaining the value of the RAP, therefore, the very marked reduction of a factor such as the ocular tension in relation to the others may account for the fact that lower RAP values than under the basal conditions were obtained, as has been observed, on the other hand, in relation to an increase of that factor.

In conclusion, therefore, even if experimental and clinical practice has shown a reduction of the endocranial pressure after glycerol and Na ascorbate, the cerebral circulation examined by ophthalmodynamometry according to Weigelin suffers no changes that could be the cause of complications consequent to the use of these substances, or if such changes have occurred, they are well compensated.

What we have mentioned in the introduction, therefore, is confirmed: The RAP values are independent of the endocranial and very probably the intraocular pressure, and only the retinal-humeral ratio is indicative of cerebral circulatory conditions.

Summary

(See p. 875 for summary in English)

Bibliography

(See p. 875-6)

Table 1. Values of Retinal Arterial Pressure (RAP), Humeral Arterial Pressure (HAP), intraocular tension before and after administration of glycerol.

I - Cases
- a - Number
- b - Name
- c - Age

II - Basal Conditions
- a - Tension
- b - HAP
- c - Mean value
- d - RAP in mm Hg
- e - Mean value

III - After glycerol (1 h)
- a - Tension
- b - HAP
- c - Mean value
- d - RAP in mm Hg
- e - Mean value

I			II					III				
a	b	c	a	b	c	d	e	a	b	c	d	e
1	C.T.	34	ODT = 12 OST = 13	125/85	102	D = 45/34 S = 49/37	39 42	ODT = 8 OST = 7	120/80	97	D = 48/34 S = 47/28	40 36
2	T.V.	32	$\overline{OOT}$ = 10	125/90	105	D = 52/32 S = 51/34	40	$\overline{OOT}$ = 7	120/90	103	D = 48/28 S = 50/29	36 38
3	L.F.	47	$\overline{OOT}$ = 16	185/130	153	D = 87/65 S = 85/63	74 72	$\overline{OOT}$ = 9	165/110	133	D = 68/56 S = 67/57	61 61
4	R.S.	50	$\overline{OOT}$ = 14	170/100	129	D = 83/55 S = 90/55	67 70	$\overline{OOT}$ = 10	170/100	129	D = 86/43 S = 82/46	61 61
5	B.A.	40	ODT = 16 OST = 17	120/80	97	D = 51/36 S = 51/36	42 42	$\overline{OOT}$ = 12	110/80	93	D = 47/33 S = 45/32	39 37
6	M.E.	62	$\overline{OOT}$ = 12	150/80	109	D = 69/40 S = 68/38	52 51	$\overline{OOT}$ = 7	150/80	109	D = 63/40 S = 63/42	50 51
7	A.P.	37	$\overline{OOT}$ = 20	130/70	95	D = 66/38 S = 66/38	50 50	$\overline{OOT}$ = 11	130/70	95	D = 61/42 S = 62/42	50 50
8	N.R.	25	$\overline{OOT}$ = 12	115/70	92	D = 52/38 S = 54/37	44 44	$\overline{OOT}$ = 8	100/70	83	D = 55/30 S = 58/84	41 42
9	A.S.	18	$\overline{OOT}$ = 14	115/70	89	D = 47/32 S = 48/32	38	$\overline{OOT}$ = 8	105/70	85	D = 44/29 S = 43/28	35 34
10	A.M.	31	$\overline{OOT}$ = 16	130/70	95	D = 64/36 S = 62/36	48 47	$\overline{OOT}$ = 10	130/70	95	D = 60/40 S = 64/38	48 49
11	A.A.	34	$\overline{OOT}$ = 12	130/90	107	D = 50/32 S = 52/34	40 42	$\overline{OOT}$ = 8	125/90	105	D = 48/26 S = 50/29	35 38
12	M.T.	46	$\overline{OOT}$ = 14	165/105	130	D = 86/53 S = 88/55	67 69	$\overline{OOT}$ = 11 OOT = 11	165/105 165/105	130 130	D = 84/43 S = 84/44	61 61

Table 2. Values of RAP, HAP, intraocular tension before and after administration of Na ascorbate.

I			II					III				
a	b	c	a	b	c	d	e	a	b	c	d	e
1	T.V.	32	OOT = 10	125/90	105	D = 60/35 S = 55/34	46 43	OOT = 6	115/85	98	D = 51/32 S = 54/34	40 42
2	R.S.	50	OOT = 14	170/100	129	D = 87/51 S = 84/49	66 64	OOT = 9	170/100	129	D = 73/44 S = 74/46	56 58
3	B.A.	40	ODT = 16 OST = 17	110/70	87	D = 50/35 S = 50/34	41 41	OOT = 12	110/70	87	D = 45/32 S = 47/32	37 38
4	M.E.	62	OOT = 12	160/90	119	D = 70/44 S = 69/45	55 55	OOT = 8	150/80	109	D = 58/38 S = 59/40	46 48
5	R.A.	61	OOT = 14	180/100	134	D = 97/46 S = 95/47	67 67	OOT = 7	180/100	134	D = 81/35 S = 83/35	54 55
6	B.L.	39	OOT = 16	115/70	89	D = 51/41 S = 50/41	45 45	OOT = 7	110/70	87	D = 48/33 S = 45/33	39 38
7	M.S.	13	OOT = 14	115/70	89	D = 47/32 S = 48/32	38 39	COT = 8	105/70	85	D = 44/29 S = 43/28	35 34
8	V.R.	15	OOT = 12	115/75	92	D = 52/38 S = 54/37	44 44	OOT = 8	100/70	83	D = 55/30 S = 53/34	41 42
9	B.C.	40	OOT = 16	180/90	145	D = 85/62 S = 84/63	72 72	OOT = 9	160/115	134	D = 66/54 S = 65/54	59 59
10	B.B.	52	OOT = 14	175/95	129	D = 85/53 S = 87/54	66 68	OOT = 10	175/95	129	D = 84/41 S = 83/44	59 60
11	R.N.	42	ODT = 15 OST = 16	120/80	97	D = 51/36 S = 51/36	42 42	OOT = 12	110/70	87	D = 45/32 S = 47/32	37 38
12	B.F.	58	ODT = 12 OST = 13	160/80	114	D = 70/42 S = 72/38	54 52	OOT = 8	160/80	114	D = 59/38 S = 60/39	47 49
13	P.P.	38	OOT = 13	130/90	107	D = 50/37 S = 52/40	42 45	OOT = 8	125/85	102	D = 48/30 S = 47/30	38 37

Table 3. Comparison between theoretical values of mean RAP according to Weigelin's data and the values recorded before and after glycerol (Values in mm HG).

No.	BEFORE		AFTER	
	Theor. values	Real values	Theor. values	Real values
1	OD = 55	39 = - 16	51	40 = - 9
	OS = 55.3	42 = - 13	50	36 = -14
2	OD = 55	40 = - 14	53	36 = -17
	OS = 55	41 = - 14	53	38 = -15
3	OD = 80	74 = - 6	66	61 = - 5
	OS = 80	72 = - 8	66	61 = - 5
4	OD = 69.6	67 = 2.6	66	61 = - 5
	OS = 69.6	70 = + 0.4	66	61 = - 5
5	OD = 53.4	42 = - 11.4	49.7	39 = -10.7
	OS = 54.5	42 = - 12.5	49.7	37 = -12.7
6	OD = 57.3	52 = - 5.3	55	50 = -5
	OS = 57.3	51 = - 6.3	55	51 = -4
7	OD = 55.6	50 = - 5.6	50.7	50 = -0.7
	OS = 55.6	50 = - 5.6	50.7	50 = -0.7
8	OD = 49.7	44 = - 5.7	43	41 = -2
	OS = 49.7	44 = - 5.7	43	42 = -1
9	OD = 48.6	38 = -10.6	43.1	34 = -9.1
	OS = 40.6	39 = - 9.6	43.1	34 = -9.1
10	OD = 54.5	48 = - 6.5	50	48 = -2
	OS = 54.5	47 = - 7.5	50	49 = - 1
11	OD = 58.1	40 = -18.1	53	38 = -15
	OS = 58.1	42 = -16.1	53	38 = -15
12	OD = 68	67 = - 1	67	61 = -6
	OS = 68	69 = + 1	67	61 = -6
	OS = 68	69 = + 1	67	61 = -6

Table 4. Comparison of the mean RAP according to Weigelin's data and the values recorded before and after Na ascorbate (values in mm Hg).

No.	BEFORE		AFTER	
	Theor. values	Real values	Theor. values	Real values
1	OD = 55	46 = 9	47.8	40 = - 7.8
	OS = 55	43 = - 12	47.8	42 = - 5.8
2	OD = 69.6	66 = - 3.6	66	56 = - 10
	OS = 69.6	64 = - 2.6	66	58 = - 8
3	OD = 49.5	41 = - 8.5	46.9	37 = - 9.9
	OS = 50	41 = - 9	46.9	38 = - 8.9
4	OD = 63.8	55 = - 8.8	54.5	46 = - 8.5
	OS = 63.8	55 = - 8.8	54.5	48 = - 6.5
5	OD - 71	67 = - 4	65.9	54 = - 11.9
	OS = 71	67 = - 4	65.9	55 = - 10.9
6	OD = 51.7	45 = - 6.6	45	39 = - 6
	OS = 51.7	45 = - 6.6	45	38 = - 7
7	OD = 50.6	38 = - 12.6	44.1	35 = - 9.1
	OS = 50.6	39 = - 11.6	44.1	34 = - 10.1
8	OD = 50	44 = - 6	43.1	41 = - 2.1
	OS = 50	44 = - 6	43.1	42 = - 1.1
9	OD = 76	72 = - 4	67.9	59 = - 8.9
	OS = 76	72 = - 4	67.9	59 = - 8.9
10	OD = 69.6	66 = - 3.6	64	59 = - 5
	OS = 69.6	68 = - 1.6	64	60 = - 4
11	OD = 54	42 = - 12	48	37 = - 11
	OS = 54.5	42 = -12.5	48	38 = - 10
12	OD = 60.8	54 = - 6.8	57.4	47 = - 10.4
	OS = 61	52 = - 9	57.4	48 = - 9.4
13	OD = 58	42 = - 16	51.7	38 = - 13.7
	OS = 58	42 = - 13	51.7	37 = - 14.7

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SUMMARY

NEUSCHÜLER R., MISSIROLI A. - *Behavior of the retinal arterial pressure in normal subjects following administration of oral glycerol and intravenous sodium ascorbate.*

After having exposed some considerations on the importance of Ophthalmodynamometry for studying the cerebral circulation, the Authors examined by means of the method of Weigelin, in 25 normal subjects, the eventual influences on cerebral circulation caused by two substances (Glycerol and Sodium ascorbate) which act electively on the cerebral bulk through an osmotic mechanism.

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CLINICA OCULISTICA DELL'UNIVERSITÀ
DEGLI STUDI DI ROMA
Direttore: Prof. G. B. BIETTI

COMPORTAMENTO DELLA PRESSIONE ARTERIOSA RETINICA IN SOGGETTI NORMALI DOPO SOMMINISTRAZIONE ORALE DI GLICEROLO ED ENDOVENOSA DI ASCORBATO DI SODIO

R. NEUSCHÜLER A. MISSIROLI

Ben noto è il fatto che l'oftalmodinamometria misurando la pressione arteriosa retinica (P.A.R.), ci dà delle precise informazioni sulle condizioni emodinamiche vigenti nelle carotidi interne. Tali vasi, essendo una delle vie di maggior afflusso ematico al cervello fanno sí che quest'ultimo risenta in maniera piú o meno sensibile le variazioni della circolazione di tale distretto, indotte dalle piú svariate cause.

Prescindendo dal comportamento della P.A.R. in fasi di diminuito calibro delle carotidi interne, è utile ricordare come la letteratura sia ricca di notizie sugli studi compiuti circa il comportamento della P.A.R. in varie condizioni fisio-patologiche o dopo assunzione di farmaci e sostanze tossiche. Anche se i risultati che sono stati segnalati non sempre sono accettabili in base alle piú recenti tecniche di misurazione e valutazione della P.A.R., la determinazione di quest'ultima, mantiene pur sempre un sostanziale valore informativo sul circolo cefalico.

Infatti un esame critico degli esperimenti compiuti, mostra come i risultati di molte ricerche non abbiano piú il valore che veniva loro attribuito, soprattutto perché non è stato tenuto nel dovuto conto, la valutazione del rapporto fra la pressione registrata al livello dell'arteria centrale della retina e di quella omerale. Già Bietti metteva in evidenza questo problema in un suo lavoro sul comportamento della P.A.R. sotto l'azione dell'adrenalina.

Dalla letteratura, soprattutto non recente, appare inoltre

come si volesse un tempo condizionare la P.A.R. non solo da parte della pressione arteriosa generale o distrettuale (come ad esempio giustamente quella esistente in una carotide rispetto all'altra); ma anche da parte della pressione intracranica (VENCO ad es. trovò, con il metodo di Baillart, una costante corrispondenza tra P.A.R. ed i vari stati di tensione intracranica), come pure che l'innalzamento del tono oculare potesse essere seguito da un aumento della P.A.R. di origine riflessa e compensatoria (VENCO 1932, 1945).

Di queste affermazioni la recente interpretazione dell'oftalmodinamometria ha fatto ormai in buona parte giustizia, ammettendo non solo che i valori pressori arteriosi rilevati al livello della papilla ottica misurano in realtà, non la pressione dell'arteria centrale retinica, ma quella esistente nell'arteria oftalmica all'incirca nel punto della sua emissione dalla carotide interna, ma anche che non hanno importanza i valori pressori retinici assoluti, ma unicamente il rapporto in cui questi stanno con la P.A.O., ed è proprio dal comportamento di questo rapporto che è possibile trarre un certo giudizio sul circolo cerebrale (LEONARDI).

Ciò, sottrae in certo modo il livello dei valori stessi da un'eventuale influenza della pressione endocranica e presumibilmente endoculare.

In relazione tuttavia ad un recente contributo (RUBICHI 1966) che ha riportato gli studi sugli effetti del glicerolo sulla P.A.R., segnalando una diminuzione dei valori dopo assunzione di tale farmaco, abbiamo voluto studiare il comportamento dei valori pressori retinici ed omerali dopo somministrazione sia di Glicerolo che di Ascorbato di Sodio. Si tratta infatti di sostanze che agiscono sulla pressione endocranica, oculare è generale, riducendone i valori in varia misura e che quindi si prestano in modo particolare a studiare se a queste variazioni possa essere sensibile l'esame oftalmodinamometrico, fornendoci così delle informazioni sulle modificazioni del circolo cerebrale eventualmente indotte.

L'uso delle suddette sostanze osmoticamente attive ha infatti suscitato particolare interesse sia nel campo della neuro-chirurgia che in oculistica, per i favorevoli risultati ottenuti nel trattamento della ipertenzione endocranica ed endoculare (VIRNO e Coll. 1963-65-66). Vogliamo ricordare a questo proposito, come già nel 1936 MARTINELLI e PAOLAZZI avessero studiato il comportamento

della P.A.R. dopo somministrazione di soluzioni ipertoniche glucosate per via endovenosa.

Questi Autori riscontrarono una diminuzione dei valori della P.A.R. a cui corrispondeva una diminuzione della pressione del liquido cefalo-rachidiano, contemporaneamente misurata.

La P.A.R. quindi, sarebbe, influenzata da sostanze osmotiche introdotte sia per via endovenosa (soluzioni glucosate ipertoniche) che per via orale (Glicerolo).

Oggi che è in corso di sperimentazione una sostanza ad azione osmotica introducibile per via endovenosa, l'Ascorbato di Sodio, e molto piú attiva del Glicerolo (infatti esplica a parità di dose un'attività superiore di 5 volte) abbiamo voluto esaminare il comportamento della P.A.R. dopo somministrazione di tale sostanza, anche in rapporto ai risultati ottenuti con l'impiego del Glicerolo.

MATERIALE E METODI

Sono stati esaminati 12 soggetti normali dopo somministrazione di Glicerolo al 50% in dose di 1 gr. pro Kilo di peso corporeo, seguendo la seguente metodica.

Si misurava la P.A.R. mediante la tecnica di Weigelin. Tale tecnica, come è noto, consiste nella misurazione della pressione diastolica e sistolica, sia retinica che omerale, da cui vengono derivate le pressioni arteriose medie secondo le indicazioni di WEZLER e BÖGER; i valori ottenuti vengono confrontati con quelli ricavati dal WEIGELIN in soggetti normali e raccolti in tabella onde stabilire le differenze dalla norma nel caso in esame.

Successivamente si somministrava il Glicerolo 50% per via orale, e dopo 1 h. si riprendevano i valori della P.A.R. e della P.A.O. Il termine di 1 h. è stato utilizzato, tenendo conto, come questo sia il momento di massima azione della sostanza, sia al livello cerebrale che oculare, sancita dalla sperimentazione.

Le misurazioni della P.A.R. sono state singole e non ripetute, onde evitare una delle cause di errore del metodo data dalla imponente caduta del tono oculare dopo ripetute compressioni del bulbo. Se eventualmente si riscontravano dei dati dubbi, si ripeteva l'esame in altra seduta.

La sperimentazione con l'Ascorbato di Na è stata eseguita su 13 soggetti, introducendo la sostanza in soluzione al 20%,

in dose di ½ gr. pro Kilo di peso corporeo, mediante fleboclisi. Anche in questo caso la P.A.R. veniva misurata con la stessa tecnica prima e dopo 1 h. dalla fine della fleboclisi, sussistendo le stesse condizioni della sperimentazione precedente.

Dall'esame della casistica si può osservare come la risposta dinamometrica dopo somministrazione delle suddette sostanze, mostri una certa diminuzione dei valori della P.A.R. nei confronti dei valori base (Tabella I e II).

Analizziamo per primi i risultati ottenuti dopo assunzione di Glicerolo (Tabella 1). I valori che abbiamo riportato, si riferiscono sia alla pressione massima e minima, che alla media delle due, ottenuta mediante la formula di Wezler e Böger (Pressione arteriosa minima a cui si aggiunge il 42% della differenziale).

Le rivelazioni sono state fatte per ciascun occhio, riscontrando delle differenze tra le due parti trascurabili sia per il valore medio che per la P.A.R. massima e minima separatamente.

Da quanto è possibile vedere la variazione dei valori della P.A.R. dopo Glicerolo (ci riferiamo alle medie) è stata in tre casi praticamente inesistente (n. 6-7-10) in cinque dai 3 ai 6 mmHg (n. 1-2-5-9-11), ed in tre superiore ai 6 fino ai 13 mmHg (n. 3-4-12). Guardando separatamente i livelli massimi e minimi, la diminuzione dei valori della P.A.R., quando si riscontrava, era percentualmente simile per le due misure peraltro con valori un po' più bassi per la P.A.R. max. Un altro dato va tenuto in conto a questo punto: il comportamento della P.A.O., che ha dimostrato una tendenza alla diminuzione dei valori, anche se modesta, in sei dei casi esaminati dove anche la P.A.R. era diminuita in vario grado; si è però anche riscontrata invariata proprio dove si sono rilevate le diminuzioni di P.A.R. più marcate (caso n. 4). La diminuzione era più sensibile nei riguardi dei suoi valori massimi e variava tra i 4 ed i 20 mmHg., considerando il valore medio. Per quanto riguarda il tono oculare, come era logico aspettarsi e come ormai ha sancito la molteplice sperimentazione in merito, ha presentato una diminuzione dei suoi valori, dopo Glicerolo, pressoché uguale nei due occhi, che variava da un minimo di 4 ad un massimo di 9 mmHg.

Analizziamo ora i valori riportati nella Tabella 2 che si riferiscono ai soggetti sottoposti a carico di Ascorbato di Na.

Anche in questi 13 casi, si osserva una certa tendenza alla diminuzione dei valori della P.A.R., senza significativa differenza tra i due lati. Il n. 8 si è mostrato scarsamente sensibile all'azione

TABELLA 1. - *Valori della P.A.R., P.A.O., oftalmotono prima e dopo somministrazione di Glicerolo.*

CASI			CONDIZIONI BASALI					DOPO GLICEROLO (1 h.)				
Numero	Nome	Età	Tono	P.A.O.	Valore medio	P.A.R. in mmHg.	Valore medio	Tono	P.A.O.	Valore medio	P.A.R. in mmHg.	Valore medio
1	*C. T.*	34	ODT = 12 OST = 13	125/85	102	PARD = 47/34 PARS = 49/37	39 42	ODT = 8 OST = 7	120/80	97	PARD = 48/34 PARS = 47/28	40 36
2	*T. V.*	32	ÕÕT = 10	125/90	105	PARD = 52/32 PARS = 51/34	40 41	ÕÕT = 7	120/90	103	PARD = 48/28 PARS = 50/29	36 38
3	*L. F.*	47	ÕÕT = 16	185/130	153	PARD = 87/65 PARS = 85/63	74 72	ÕÕT = 9	165/110	133	PARD = 68/56 PARS = 67/57	61 61
4	*R. S.*	50	ÕÕT = 14	170/100	129	PARD = 83/55 PARS = 90/55	67 70	ÕÕT = 10	170/100	129	PARD = 86/43 PARS = 82/46	61 61
5	*B. A.*	40	ODT = 16 OST = 17	120/80	97	PARD = 51/36 PARS = 51/36	42 42	ÕÕT = 12	110/80	93	PARD = 47/33 PARS = 45/32	39 37
6	*M. E.*	62	ÕÕT = 12	150/80	109	PARD = 69/40 PARS = 68/38	52 51	ÕÕT = 7	150/80	109	PARD = 63/40 PARS = 63/42	50 51
7	*A. P.*	37	ÕÕT = 20	130/70	95	PARD = 66/38 PARS = 66/38	50 50	ÕÕT = 11	130/70	95	PARD = 61/42 PARS = 62/42	50 50
8	*N. R.*	25	ÕÕT = 12	115/70	92	PARD = 52/38 PARS = 54/37	44 44	ÕÕT = 8	100/70	83	PARD = 55/30 PARS = 53/84	41 42
9	*A. S.*	18	ÕÕT = 14	115/70	89	PARD = 47/32 PARS = 48/32	38 39	ÕÕT = 8	105/70	85	PARD = 44/29 PARS = 43/28	35 34
10	*A. M.*	31	ÕÕT = 16	130/70	95	PARD = 64/36 PARS = 62/36	48 47	ÕÕT = 10	130/70	95	PARD = 60/40 PARS = 64/38	48 49
11	*A. A.*	34	ÕÕT = 12	130/90	107	PARD = 50/32 PARS = 52/34	40 42	ÕÕT = 8	125/90	105	PARD = 48/26 PARS = 50/29	35 38
12	*M. T.*	46	ÕÕT = 14	165/105	130	PARD = 86/53 PARS = 88/55	67 69	ÕÕT = 11 ÕÕT = 11	165/105 165/105	130 130	PARD = 84/43 PARS = 84/44	61 61

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TABELLA 2. - *Valori della P.A.R., P.A.O., oftalmotono prima e dopo somministrazione di Ascorbato di Na.*

CASI			CONDIZIONI BASALI					DOPO ASCORBATO DI Na (1 h.)				
Numero	Nome	Età	Tono	P.A.O.	Valore medio	P.A.R. in mmHg.	Valore medio	Tono	P.A.O.	Valore medio	P.A.R. in mmHg.	Valore medio
1	*T. V.*	32	$\overline{O}\overline{O}T = 10$	125/90	105	PARD = 60/35 PARS = 55/34	46 43	$\overline{O}\overline{O}T = 6$	115/85	98	PARD = 51/32 PARS = 54/34	40 42
2	*R. S.*	50	$\overline{O}\overline{O}T = 14$	170/100	129	PARD = 87/51 PARS = 84/49	66 64	$\overline{O}\overline{O}T = 9$	170/100	129	PARD = 73/44 PARS = 74/46	56 58
3	*B. A.*	40	ODT = 16 OST = 17	110/70	87	PARD = 50/35 PARS = 50/34	41 41	$\overline{O}\overline{O}T = 12$	110/70	87	PARD = 45/32 PARS = 47/32	37 38
4	*M. E.*	62	$\overline{O}\overline{O}T = 12$	160/90	119	PARD = 70/44 PARS = 69/45	55 55	$\overline{O}\overline{O}T = 8$	150/80	109	PARD = 58/38 PARS = 59/40	46 48
5	*R. A.*	61	$\overline{O}\overline{O}T = 14$	180/100	134	PARD = 97/46 PARS = 95/47	67 67	$\overline{O}\overline{O}T = 7$	180/100	134	PARD = 81/35 PARS = 83/35	54 55
6	*B. L.*	39	$\overline{O}\overline{O}T = 16$	115/70	89	PARD = 51/41 PARS = 50/41	45 45	$\overline{O}\overline{O}T = 7$	110/70	87	PARD = 48/33 PARS = 45/33	39 38
7	*M. S.*	13	$\overline{O}\overline{O}T = 14$	115/70	89	PARD = 47/32 PARS = 48/32	38 39	$\overline{O}\overline{O}T = 8$	105/70	85	PARD = 44/29 PARS = 43/28	35 34
8	*V. B.*	15	$\overline{O}\overline{O}T = 12$	115/75	92	PARD = 52/38 PARS = 54/37	44 44	$\overline{O}\overline{O}T = 8$	100/70	83	PARD = 55/30 PARS = 53/34	41 42
9	*R. C.*	40	$\overline{O}\overline{O}T = 16$	180/90	145	PARD = 85/62 PARS = 84/63	72 72	$\overline{O}\overline{O}T = 9$	160/115	134	PARD = 66/54 PARS = 65/54	59 59
10	*B. B.*	52	$\overline{O}\overline{O}T = 14$	175/95	129	PARD = 85/53 PARS = 87/54	66 68	$\overline{O}\overline{O}T = 10$	175/95	129	PARD = 84/41 PARS = 83/44	59 60
11	*R. N.*	42	ODT = 15 OST = 16	120/80	97	PARD = 51/36 PARS = 51/36	42 42	$\overline{O}\overline{O}T = 12$	110/70	87	PARD = 45/32 PARS = 47/32	37 38
12	*B. F.*	58	ODT = 12 OST = 13	160/80	114	PARD = 70/42 PARS = 72/38	54 52	$\overline{O}\overline{O}T = 8$	160/80	114	PARD = 59/38 PARS = 60/39	47 48
13	*P. P.*	38	$\overline{O}\overline{O}T = 13$	130/90	107	PARD = 50/37 PARS = 52/40	42 45	$\overline{O}\overline{O}T = 8$	125/85	102	PARD = 48/30 PARS = 47/30	38 37

dell'Ascorbato di Sodio, mentre per gli altri casi si sono avute variazioni in meno dei valori P.A.R. dai 3 ai 6 mmHg. (n. 1-3-7-11) ed anche fino a 13 mmHg (n. 9). Tali valori si riferiscono alla P.A.R. media e se poi si analizzano separatamente la P.A.R. massima e minima, si vede, come si è già osservato per i casi trattati con Glicerolo, che la diminuzione dei due valori è percentualmente simile, con una prevalente diminuzione per i valori massimi. La P.A.O. è rimasta invariata in cinque casi, in uno dei quali (n. 5) si è riscontrata una corrispettiva diminuzione della P.A.R. evidente; negli altri è diminuita dai 5 ai 10 mmHg. per i valori medi, e piú marcatamente la massima che la minima.

L'oftalmotono, come anche nei casi trattati con Glicerolo, ha mostrato una significativa diminuzione dei suoi valori (dai 4 ai 9 mmHg.) uguale nei due occhi.

A questo punto però, l'esame dei dati ottenuti mediante le tabelle di WEIGELIN, mostra che nonostante il valore della P.A.R. considerato isolatamente, evidenzi una piú o meno indicativa diminuzione dopo assunzione delle sostanze osmotiche adoperate, il rapporto retino-omerale, unico parametro da cui si possa trarre un giudizio sul circolo sanguigno intracranico, rimane generalmente nei limiti della norma indicati dal metodo (+ o — 6 mmHg di differenza dal valore teorico indicato dalle tabelle), come è possibile vedere dalle tabelle n. 3 e 4.

CONSIDERAZIONI E CONCLUSIONI

Come abbiamo detto nell'introduzione, scopo della nostra indagine è stato quello di studiare le eventuali modificazioni oftalmodinamometriche indotte da due sostanze osmoticamente attive: Glicerolo ed Ascorbato di Na. Ordunque, stanti i presupposti che hanno dimostrato clinicamente e sperimentalmente come dato inconfutabile il grande potere disidratante e pertanto riduttore della massa cerebrale e della pressione endocranica, nonché dell'oftalmotono, come anche della pressione generale, da parte delle due sostanze usate, ed esaminando isolatamente i valori della P.A.R. in senso assoluto, si potrebbe essere erroneamente indotti a credere in una vasodilatazione del distretto cefalico secondaria a diminuita tensione della massa cerebrale disidratata, quindi con una circolazione intracranica facilitata.

Questo in realtà non è vero, infatti l'esame del rapporto retino-

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TABELLA 3. - *Tabella di confronto tra valori teorici della P.A.R. media secondo i dati di Weigelin ed i valori registrati prima e dopo Glicerolo. (I valori sono in mmHg).*

Numero	PRIMA		DOPO	
	Valori teorici	Valori reali	Valori teorici	Valori reali
1	OD = 55	39 = — 16	51	40 = — 9
	OS = 55,3	42 = — 13	50	36 = — 14
2	OD = 55	40 = — 15	53	36 = — 17
	OS = 55	41 = — 14	53	38 = — 15
3	OD = 80	74 = — 6	66	61 = — 5
	OS = 80	72 = — 8	66	61 = — 5
4	OD = 69,6	67 = 2,6	66	61 = — 5
	OS = 69,6	70 = + 0,4	66	61 = — 5
5	OD = 53,4	42 = — 11,4	49,7	39 = — 10,7
	OS = 54,5	42 = — 12,5	49,7	37 = — 12,7
6	OD = 57,3	52 = — 5,3	55	50 = — 5
	OS = 57,3	51 = — 6,3	55	51 = — 4
7	OD = 55,6	50 = — 5,6	50,7	50 = — 0,7
	OS = 55,6	50 = — 5,6	50,7	50 = — 0,7
8	OD = 49,7	44 = — 5,7	43	41 = — 2
	OS = 49,7	44 = — 5,7	43	42 = — 1
9	OD = 48,6	38 = — 10,6	43,1	35 = — 8,1
	OS = 40,6	39 = — 9,6	43,1	34 = — 9,1
10	OD = 54,5	48 = — 6,5	50	48 = — 2
	OS = 54,5	47 = — 7,5	50	49 = — 1
11	OD = 58,1	40 = — 18,1	53	35 = — 18
	OS = 58,1	42 = — 16,1	53	38 = — 15
12	OD = 68	67 = — 1	67	61 = — 6
	OS = 68	69 = + 1	67	61 = — 6
	OS = 68	69 = + 1	67	61 = — 6

omerale, unica maniera, come abbiamo detto, per avere una reale valutazione del circolo cefalico secondo la tecnica di Weigelin, non evidenzia alcun squilibrio tra i valori della pressione generale e retinica, costatando al contrario una generale sovrapponibilità

TABELLA 4. - *Tabella di confronto della P.A.R. media secondo i dati di Weigelin ed i valori registrati prima e dopo Ascorbato di Na. (I valori sono in mmHg).*

Numero	PRIMA		DOPO	
	Valori teorici	Valori reali	Valori teorici	Valori reali
1	OD = 55	46 = 9	47,8	40 = — 7,8
	OS = 55	43 = — 12	47,8	42 = — 5,8
2	OD = 69,6	66 = — 3,6	66	56 = — 10
	OS = 69,6	64 = — 2,6	66	58 = — 8
3	OD = 49,5	41 = — 8,5	46,9	37 = — 9,9
	OS = 50	41 = — 9	46,9	38 = — 8,9
4	OD = 63,8	55 = — 8,8	54,5	46 = — 8,5
	OS = 63,8	55 = — 8,8	54,5	48 = — 6,5
5	OD = 71	67 = — 4	65,9	54 = — 11,9
	OS = 71	67 = — 4	65,9	55 = — 10,9
6	OD = 51,7	45 = — 6,6	45	39 = — 6
	OS = 51,7	45 = — 6,6	45	38 = — 7
7	OD = 50,6	38 = — 12,6	44,1	35 = — 9,1
	OS = 50,6	39 = — 11,6	44,1	34 = — 10,1
8	OD = 50	44 = — 6	43,1	41 = — 2,1
	OS = 50	44 = — 6	43,1	42 = — 1,1
9	OD = 76	72 = — 4	67,9	59 = — 8,9
	OS = 76	72 = — 4	67,9	59 = — 8,9
10	OD = 69,6	66 = — 3,6	64	59 = — 5
	OS = 69,6	68 = — 1,6	64	60 = — 4
11	OD = 54	42 = — 12	48	37 = — 11
	OS = 54,5	42 = — 12,5	48	38 = — 10
12	OD = 60,8	54 = — 6,8	57,4	47 = — 10,4
	OS = 61	52 = — 9	57,4	48 = — 9,4
13	OD = 58	42 = — 16	51,7	38 = — 13,7
	OS = 58	42 = — 13	51,7	37 = — 14,7

ci sia stata una modificazione del circolo cerebrale, o questa è stata ben compensata dal circolo generale, oppure che le modificazioni dei vari parametri necessari alla valutazione della P.A.R. eventualmente [illegible] di compensamento

parte è ormai noto, sono molto significative. Tant'è vero che ci siamo trovati spesso di fronte a toni oculari che sotto l'azione delle sostanze usate, presentavano dei valori molto al di sotto di quelli minimi previsti nelle tabelle usate per il calcolo della P.A.R.

Probabilmente quindi nella formulazione dei calcoli necessari per ottenere il valore della P.A.R., la diminuzione così marcata di un fattore come il tono oculare in rapporto agli altri, come d'altra parte è stato osservato nei confronti di un suo aumento, può essere stato determinante agli effetti di ottenere dei valori P.A.R. più bassi che nelle condizioni di base.

Concludendo quindi, anche se la pratica sperimentale e clinica hanno dimostrato una diminuzione della pressione endocranica dopo Glicerolo ed Ascorbato di Na, il circolo cerebrale studiato mediante l'oftalmodinamometria secondo Weigelin, non subisce modificazioni che possano eventualmente essere causa di complicazioni secondarie all'uso di tali sostanze, o per lo meno che tali modificazioni, qualora si siano verificate, sono state bene compensate.

Una riprova pertanto, a quanto abbiamo accennato nell'introduzione: l'indipendenza dei valori della P.A.R. dalla pressione endocranica e con molta probabilità endoculare e che solo il valore del rapporto retino-omerale, è idoneo ad indicarci le condizioni circolatorie cerebrali.

RIASSUNTO

Gli AA., dopo alcune considerazioni in merito all'importanza dell'oftalmodinamometria per lo studio del circolo cerebrale, hanno voluto studiare su 25 soggetti normali, mediante il metodo di Weigelin, le eventuali influenze su di esso provocate, da due sostanze che agiscono elettivamente sulla massa cerebrale con meccanismo osmotico (Glicerolo ed Ascorbato di Na).

RÉSUMÉ

NEUSCHÜLER R., MISSIROLI A. - *Comportement de la tension artérielle rétinienne chez les sujets normaux après administration du glycérol par voie buccale et de l'Ascorbate de soude par voie intraveineuse.*

Après avoir pris en considération l'importance de l'Ophtalmodynamometrie pour l'étude de la circulation cérébrale, les Auteurs ont voulu examiner, au moyen de la méthode de Weigelin, chez 25 sujets normaux les éventuelles influences sur la circulation cérébrale expliquées par deux substances (le Glycérol et l'Ascorbate de soude) qui agissent élect-ivemente sur la masse cérébrale avec un mécanisme osmotique.

SUMMARY

NEUSCHÜLER R., MISSIROLI A. - *Behavior of the retinal arterial pressure in normal subjects following administration of oral glycerol and intravenous sodium ascorbate.*

After having exposed some considerations on the importance of Ophthalmodynamometry for studying the cerebral circulation, the Authors examined by means of the method of Weigelin, in 25 normal subjects, the eventual influences on cerebral circulation caused by two substances (Glycerol and Sodium ascorbate) which act electively on the cerebral bulk through an osmotic mechanism.

ZUSAMMENFASSUNG

NEUSCHÜLER R., MISSIROLI A. - *Das Verhalten des Netzhautblutdruckes in normalen Subjekten nach der Verabreichung von peroralen Glyzerol und intravenöses Natrium Askorbinat.*

Nach einigen Überlegungen über die Bedeutung der Ophthalmodynamometrie zur Untersuchung des Verhaltens des zerebralen Blutkreislaufes, Blutkreislaufes, haben die Verfasser in normalen Subjekten, mitels der Methode von Weigelin, die möglichen Einflüsse auf den zerebralen Blutkreislauf, welche von zwei Substanzen (Glyzerol und Natrium Askorbinat), die mit einem osmotischen Mechanismus spezifisch auf der zerebralen Masse wirken, verursacht werden studiert.

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POSSIBILITÀ TERAPEUTICHE DELL'ASSOCIAZIONE GLICEROLO-ACIDO ASCORBICO PER VIA ORALE, NEL TRATTAMENTO DEL GLAUCOMA

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L'impiego di sostanze ipotonizzanti oculari ad azione osmotica introdotte per via orale e per via endovenosa nel trattamento delle varie forme di glaucoma, è una acquisizione nota sia in campo sperimentale che clinico. Tali sostanze, tra le quali l'urea, il mannitolo e il glicerolo, hanno la proprietà di aumentare l'osmolarità ematica, richiamando in tal modo acqua dai vari distretti dell'organismo, specialmente da quelli maggiormente idratati, ed in particolar modo dal globo oculare (1).

Per quanto riguarda il glicerolo, è noto che tale sostanza, somministrata per via orale in soluzione acquosa al 50%, riduce in modo rapido e marcato l'oftalmotono sia di occhi normali che glaucomatosi (9, 10).

L'azione del glicerolo comincia ad evidenziarsi dopo 20-30 minuti dalla somministrazione, ed i massimi valori di ipotonia vengono raggiunti dopo 90-120 minuti e si mantengono per 4-5 ore circa.

L'azione del glicerolo è dovuta esclusivamente ad un meccanismo osmotico, come è stato dimostrato da TREVOR-ROPER (4).

Altra sostanza, recentemente studiata come mezzo ipotensivo oculare presso la nostra Clinica, è la Vitamina C che, per le sue peculiari caratteristiche, non si può completamente ascrivere al gruppo degli agenti osmotici (3).

La Vitamina C viene attualmente usata sotto due forme: per infusione endovenosa (ascorbato di sodio in soluzione al 20% a pH 7.2-7.4) e per via orale (acido ascorbico in soluzione al 20% a pH 2.1) (5, 6, 7, 8).

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THE EFFECT OF ASCORBIC ACID (VITAMIN C), CALCIUM ASCORBATE, AND CALCIUM GLUCONATE ON THE REGENERATION OF BONE IN RATS.

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THERE is abundant evidence in the literature that vitamin C is associated with the production and maintenance of collagen fibres [Watanabe, 1924; Wolbach and Howe, 1925, 1926; Mazoué, 1937; Jéney and Törö, 1938; Quérido and Gaillard, 1939; Hunt, 1941].

Since bone possesses a fibrous collagenous-like matrix it is reasonable to expect an imperfect regeneration of bone in scorbutic animals. This has been shown to be a fact [Ferraris and Lewi, 1923; Israel and Frankel, 1926; Kappis, 1927; Schilozew, 1928; Salter and Aub, 1931; Roegholt, 1932; Jéney and Korpàssy, 1934; Hertz, 1936; Ham and Elliott, 1938; Klein, 1939].

This experimental work is supported by numerous clinical observations [*e.g.* Mead, 1762; Marrigues, 1783; Collisen, 1798; Hammick, 1830; Budd, 1840; Moore, 1859; Lobmayer, 1918, etc.].

The next question to be considered is whether the regeneration of bone can be accelerated by the administration of amounts of vitamin C in excess of that needed to saturate the organism with the vitamin. It seems unlikely that this would be so, because excess vitamin C is rapidly excreted in the urine.

Halàsz and Marx [1932] appear to have first studied this problem in guinea-pigs. They fed their animals on a diet which was rich in vitamin C, and in addition they administered lemon juice daily with a pipette. They found no radiographic or microscopic evidence that the healing of the fracture was accelerated. On the other hand, Lexer [1939] found that in three groups of guinea-pigs which received (i) a normal diet, (ii) a normal diet and extra vitamin C, and (iii) a diet deficient in vitamin C, the healing of fractures as determined by X-ray examination was best in group (ii) and worst in group (iii).

Hanke [1935] found that vitamin C injected into rabbits (which are usually regarded as being able to synthesise the vitamin and

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therefore as being saturated with it) accelerated bone regeneration after resection. He found also that rabbits on a vitamin C free diet eventually regenerated resected bone, but did so only very slowly.

Giangrasso [1939 *a*] found that daily subcutaneous injections of 10 mg. of vitamin C into rabbits with a fractured forearm caused an increase in the rate of healing (radiographic and microscopic evidence). In another series of experiments [1939 *b*] he found injection of vitamin C more effective than injections of vitamin D. In a third paper [1939 *c*] he suggested that large doses of vitamin C (100 mg. a day) slightly retarded healing in the beginning, but that this action rapidly passed off.

None of the authors who have investigated the influence of extra-saturation vitamin C on fracture healing appear to have subjected their results to a statistical analysis, or indeed to have used methods which lent themselves to such an analysis, although Hanke has published some rather convincing X-ray photographs. An attempt has been made in the experiments recorded here to obtain an accurate estimation of the ability of various substances to accelerate bone regeneration.

Technique.

Varying techniques have been used for studying the regeneration of bone. In general, fracture of a bone, whether it be fracture of the femur [Israel and Frankel, 1926; Schilozew, 1928; Halàsz and Marx, 1932], fracture of the radius and/or ulna [Giangrasso, 1939], fracture of metatarsal bones [Otsuki, 1927; Emori, 1927], or fracture of the ribs [Moritsch and Krammer, 1929; Ferraris and Lewi, 1923; and Emori, 1927], is unsatisfactory for an accurate comparative study of the regeneration of bone. The reasons for this are that it is impossible to control the amount of trauma caused by each fracture, and if an injured limb has to be kept in splints or plaster to support it, and to keep the broken ends in apposition, these are rapidly gnawed off by the animals. Hertz [1936] has controlled most of these variables by the introduction of the fracture forceps and by its use to fracture the fibula of rats and guinea-pigs. One advantage of this method is that the leg does not require splinting, because the tibia supports the broken bone and the ends of the fractured bone are kept more or less in alignment by the surrounding soft tissues. Even so, it must be difficult to control adequately the trauma and the alignment of the broken ends, as well as the nature of the break.

Other workers have, by aseptic techniques, sawn through bones; and Vara Lopez [1928], in the case of an ulna treated in this way, rendered the two ends of the sawn bone immovable by suturing the limb into the muscles of the chest wall. Watanabe [1924] sawed a small fissure in the cranium, and Wolbach and Howe [1925, 1926] used a small circular saw to produce fissures in the femur of the guinea-pig.

It is difficult by these methods, however, to obtain fissures of the same size. It was decided in these experiments, in order to obtain exactly comparable areas for regeneration to take place, to use a dentist's twist drill of standard size and to bore in each case through the cortex of the bone into the medulla. Thus in a few minutes it was possible to obtain a series of lesions of identical size and shape.

The animals used in the present experiments were white rats of Glaxo, or Glaxo descended, stock, their body-weights varied between 70 and 130 g. Twenty-four hours before operation the hair from the whole of the hinder portion of the body, and of the outer surfaces of the hind legs, was removed with the aid of barium sulphide. Immediately prior to the operation the skin so exposed was painted with tincture of iodine or with a 1/1000 solution of acriflavine, and just before an incision was made the skin was washed with ether.

The rats were anæsthetised with nembutal, and were secured on a specially designed operating board which consisted of a rectangular block of wood fastened upon a flat board. The hind quarters of the animal were placed upon the block. The legs were secured by cords attached to two screws placed behind and to the side of the block. The thorax of the animal was held firmly to the flat board by a piece of cloth with drawing-pins. In such a position the animal was firmly held and the block acted as a support for the femurs whilst they were being drilled. Once secured on this board, the rat was placed in such a position that by making a skin incision in each leg, snipping through the subcutaneous connective tissue, and pushing the muscles to one side, the femur can be exposed. There was a negligible amount of bleeding at this stage. An electrically driven, 1 mm. diameter, dentist's twist drill was used to bore a hole approximately in the centre of the shaft of each femur. Penetration of the cortex of the bone resulted in a hæmorrhage from the marrow, but a small piece of cotton-wool left in contact with the hole for a few seconds after drilling stopped the bleeding. A single stitch of silk thread served to secure the fascia, and the skin wound was closed with one or two "Michel" clips, which were removed 3 days after the operation. The whole operation was performed using strict aseptic precautions, because, although the rat generally is not susceptible to pyogenic infections, bone does appear to be more easily infected than other rat tissues. In two cases slight suppuration of the skin wound was observed, probably due to post-operative infection, but in no case was any infection of the bone found to be present.

Immediately following the operation the animals were injected with the various test substances, and the injections were continued daily. Controls were given normal saline. After operation and first injection the animals, while still under the influence of nembutal, were wrapped in cotton-wool and placed together in a box with hot-water

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bottles until the next morning. They were given only water to drink during this period. The following morning they were placed, each in a separate cage. Each cage was of identical size, so that all the rats were able to take the same amount of exercise. Each animal received as much as it could eat daily of rat cake, milk, greens, and carrots, or, if these last two were not available, of dried alfalfa. Two drops of halibut liver oil were also given daily to each rat. Weighings of the amounts of food eaten by each rat showed that they consumed approximately the same amount of the various substances given, and it was therefore assumed that the dietary intake of calcium was approximately the same in all animals.

Usually five animals were operated on at the one time and each was given a different treatment. One was injected daily with vitamin C, one with calcium glucono-galacto-gluconate, a third with calcium ascorbate (a calcium salt of vitamin C), a fourth, in some experiments only, with separate solutions of vitamin C and calcium glucono-galacto-gluconate, and a fifth with normal saline. By distributing the animals of each series operated on throughout the experimental groups, any uncontrolled, or uncontrollable, variations which occurred at the time and which might have weighted the results towards any particular group were prevented.

All test substances were injected subcutaneously.

The following are the body-weights of the animals of the various experimental groups. There is no significant difference between any of them.

BODY-WEIGHTS.

Controls.	Ca ascorbate.	Ca gluconate.	Vitamin C.	Vitamin C and Ca gluconate.
85·8 ± 8·0	93·0 ± 4·5	85·3 ± 8·2	99·0 ± 9·6	97·0 (three animals only)

The Ca ascorbate group received 50 mg. of Ca ascorbate a day. The vitamin C group received the same amount of vitamin C as was contained in 50 mg. of Ca ascorbate, *i.e.* about 45 mg. The Ca glucono-galacto-gluconate group received an amount of calcium equal to that present in the calcium ascorbate; namely, about 5 mg. The group in which vitamin C and calcium glucono-galacto-gluconate and vitamin C were injected in separate solutions received the same amounts of these substances as were contained in the Ca ascorbate—that is, 45 mg. of vitamin C and 5 mg. of calcium.

Most of the animals used for this work were kept for 1 week after the operation. Other experiments were performed in which the animals were kept for 3 and for 4 days.

At the conclusion of the experimental period the animals were killed with chloroform, the femurs were rapidly dissected out and fixed in 10 per cent. acetic acid containing 10 per cent. silver nitrate in dark brown bottles for 12 hours. This reagent was used because it was hoped to use them later for a cytological study of the distribution of vitamin C in the callus. After 12 hours the femurs were removed from the fixative, washed for an hour in distilled water, and then placed in 10 per cent. trichloracetic acid for 36 hours in order to decalcify them. They were then washed, dehydrated, and embedded in wax for standard periods of time. The use of standard times for these processes is essential, otherwise one gets variable shrinkage of the trabeculæ which have formed. In addition, all manipulation of the femurs from fixing to embedding was carried out in standard-sized bottles, one femur to each bottle, and a standard volume of fluid was used in each case. Serial sections were made through the hole which had been bored in the femur and the sections were stained with hæmatoxylin and van Gieson stains.

The work recorded in this paper deals with only one phase of the healing process, namely, the formation of temporary membrane bone. No attempt has been made as yet to investigate the effects of any of the substances injected on the formation of cartilage, on endochondral ossification, or on the subsequent remoulding process. Observation of the healing process at various times after the boring of the hole were made in order to ascertain when would be the most suitable time to estimate the degree of healing which had taken place. The amount of bony regeneration which had occurred in such a hole was followed by killing animals at 3 days, 4 days, 7 days, 11 days, 14 days, and 18 days after the operation. Immediately the hole has been bored there is a hæmorrhage from the medulla. The blood, however, clots in a few seconds. Two days after the operation, numerous fibroblasts are present in the clot and fine fibres ramify through it. These fibres do not stain with van Gieson and are probably composed of precollagen. By 4 days, strands of van Gieson staining material make their appearance, and finally appear to form themselves into anastomosing and branching trabeculæ. This stage has been reached by 7 days. This van Gieson staining material is ossein; it is the equivalent, in bone, of connective tissue collagen. Between 7 days and 18 days (the latest stage of healing investigated in this work) the changes consist chiefly of the further growing over and thickening of the periosteum and the development of cartilage in the callus. Some specimens show what appears to be cartilage formed by the periosteum at 4 days, but it is not until very late (*e.g.* around 18 days) that any appreciable amount of cartilage appears in the callus.

Since by 7 days an appreciable number of bony trabeculæ had been formed in the region of the hole, this time was taken as the

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most suitable for killing the animals. An estimate of the amount of trabeculæ formed was obtained in the following way.

The slide bearing the sections through the hole in the femur was placed in a projector and the images of the trabeculæ (magnification × 100) present in a standard rectangular area were traced on to paper (see fig. 1). This was repeated in five separate sections of each femur, each section being taken at random near, or at, the centre of the hole. The standard rectangle of paper on which the trabeculæ had been traced

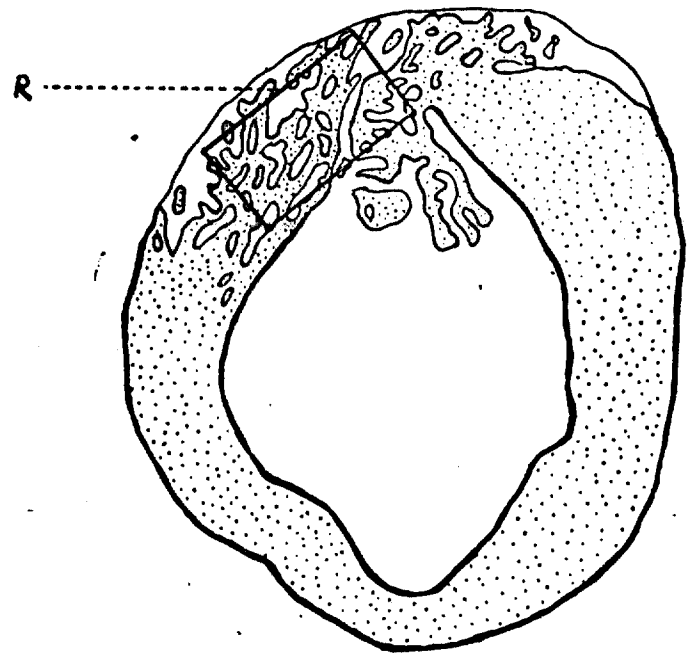

FIG. 1.—Projection drawing (× 32) of a transverse section through the femur of a rat. The section passes through the centre of a hole which has been drilled one week before. The rectangle of paper ("R") which includes the trabeculæ is weighed. The trabeculæ are cut out and weighed. The second weight divided by the first gives the "trabecular index."

was then weighed. Let its weight equal W. With a pair of curved scissors the trabeculæ were then cut out and weighed (let weight equal T). Then T/W gives an index of the amount of bone regenerated in the hole. This may be described as the "trabecular index" of regeneration. By means of this method it has been possible to obtain a series of figures which could be subjected to statistical examination.

Since rats synthesise their own vitamin C and can therefore be assumed to be saturated with the vitamin, they were therefore regarded as ideal animals for testing out by this method the effect of extra-saturation vitamin C on regeneration of bone.

Calcium ascorbate was tried as an accelerator for two reasons. In the first place, it has been shown by Urist and McLean [1941] that the

conception of an organic matrix being first laid down by regenerating bone, and this being later followed by the deposition of bone salts, is incorrect. According to them the organic matrix and the bone salt are laid down simultaneously, and if there is a delay in the deposition of bone salt it is an abnormality, and is "almost to be classed as a minimal form of rickets." Urist and McLean found that it occurred in some litter mates on the same diet and not in others. They suggest that it is due to an "insufficient local supply of bone minerals." Since vitamin C plays an important part in the formation of organic bone matrix and since calcium is an essential constituent of bone salt, it was considered that calcium ascorbate would be worth trying as an accelerator of bone healing. Secondly, the work of Ruskin [1938] suggested that calcium ascorbate was an ideal form in which to administer calcium. He claims that calcium gluconate is only 3 per cent. soluble in water and that Ca ascorbate is almost 100 per cent. soluble. The Ca ion content of a 3 per cent. solution of Ca gluconate he found to be 0·0014, and that of a 30 per cent. solution of Ca ascorbate was found to be 0·0226. This greater degree of ionisation suggested that Ca ascorbate would be more easily absorbed by the body tissues.

To ensure that any effect of the Ca ascorbate on the healing process was due to the injection of Ca as the ascorbate, a small number of animals were injected at the same time with separate solutions of vitamin C and Ca glucono-galacto-gluconate.

The compound calcium glucono-galacto-gluconate is produced by Sandoz and is a product which has improved solubility over the older Ca gluconate. The new product can, according to the makers, be obtained in a 30 per cent. solution. No Ca ion content for the various concentrations is published.

Results.

The following tables summarise the results obtained in this work. The trabecular index shown is an average figure obtained from five sections taken through the central region of the hole in each femur. That is to say, each index represents the average of ten samples.

Trabecular Indices.

Controls. Normal saline.	Ca ascorbate.
0·40	0·54
0·47	0·54
0·45	0·52
0·47	0·55
0·33	0·57
0·32	0·55
0·38	0·49
0·42	0·46
0·38	0·51
Mean = 0·402 ± 0·0185	Mean = 0·526 ± 0·0115
$n = 9$	$n = 9$

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TRABECULAR INDICES—*Continued.*

Ca g.-g.-gluconate.	Vitamin C.
0·47	0·46
0·44	0·36
0·46	0·50
0·44	0·52
0·44	0·47
0·47	0·37
Mean = 0·453 ± 0·0069	Mean = 0·447 ± 0·0273
$n = 6$	$n = 6$

The following table summarises the results of the application of Fisher's "t" test for significance to these figures:—

	Ca ascorbate.	Vitamin C.	Ca g.-g.-gluconate.
Controls	$t = 5·698$ P < 0·01	$t = 1·423$ P lies between 0·2 and 0·1	$t = 2·164$ P lies between 0·05 and 0·02
Ca ascorbate		$t = 3·046$ P < 0·01	$t = 4·807$ P < 0·01
Vitamin C			$t = 0·2147$ P lies between 0·9 and 0·8

These results indicate that calcium ascorbate produces significantly more bone than that formed in the controls or in those animals which were injected with vitamin C or Ca g.-g.-gluconate. The amount of bone formed in the vitamin C injected group does not differ significantly from that produced by the controls or the calcium g.-g.-gluconate injected group. The latter group produces more bone than the controls, and the amount formed approaches a significant difference. Possibly if there were a greater number of animals in this group the difference may become significant. It is interesting that the calcium ascorbate group have produced more than the Ca g.-g.-gluconate group, because gluconic acid, although without antiscorbutic power, is closely related chemically to ascorbic acid.

Three animals were injected with Ca g.-g.-gluconate and vitamin C in separate solutions, and they gave the following results:—

TRABECULAR INDEX.

	0·25
	0·27
	0·15
Mean	0·223

This is less than half the amount of bone formed by the ascorbic acid group, and only a little more than half the amount formed by the controls. It is difficult to imagine that these two substances injected in separate solutions into the same animal really do retard bone healing. Nevertheless the three results represent those obtained in three separate experiments. They were all rats which had been members of separate groups of five which had been operated on at the same time and injected with different substances; the results given by the other members of the three groups are shown below for comparison:

Vitamin C and Ca g.-g.-gluconate.	Ca ascorbate.	Vitamin C.	Ca g.-g.-gluconate.	Control. N. saline.
0·25	0·54	0·46	0·47	0·45
0·27	0·55	0·36	0·46	0·47
0·15	0·52	0·52	0·44	0·47

It is obvious that this matter needs further investigation.

Attempts have been made to estimate the effects of calcium ascorbate at earlier stages of the healing process, that is at 3 days and at 4 days. Four animals were injected with normal saline as controls and four were injected with calcium ascorbate for the 3-day experiments. The results are tabulated below:

THREE DAYS. TRABECULAR INDEX.

	Controls.		Ca ascorbate.
	0·066		0·066
	0·026		0·077
	0·057		0·023
	0·071		0·065
Mean	0·055 ± ·010	Mean	0·058 ± ·011

There is obviously no significant difference between these results.

Four animals were used in the 4-day experiment, two controls injected with normal saline, and two animals injected with calcium ascorbate.

FOUR DAYS. TRABECULAR INDEX.

	Controls.		Ca ascorbate.
	0·097		0·059
	0·032		0·060
Mean	0·065	Mean	0·060

This result is obviously not significant.

The results of the 3- and 4-day experiments (although from only a small number of animals) suggest that if Ca ascorbate has an effect

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on the healing of fractures at a very early stage, the technique used is not delicate enough to detect it, or an insufficient number of animals was used in the experiments. Presumably, however, the effects of the Ca ascorbate injections are cumulative, and after a week they are sufficiently advanced to enable a statistical difference to be detected.

It is possible that the injections of vitamin C, in the 7-day experiments, are slow in bringing about an effect, and that if the injections had been continued for a week longer a statistical increase in the amount of bone formed might have been seen in this group also. The literature, however, suggests an early effect of vitamin C injections. Gingrasso, in fact, claims that in rabbits vitamin C exerts an effect as early as 3 days after the first injection.

Discussion.

The results given in this paper show that vitamin C in the amounts injected does not accelerate the healing of bone in an animal such as the rat, which is normally saturated with this vitamin. They do show, however, that subcutaneous injections of calcium ascorbate are more effective than subcutaneous injections of either vitamin C or of calcium glucono-galacto-gluconate in accelerating the regeneration of bone in rats. These experiments do not of themselves give any indication whether subcutaneous injection of calcium ascorbate is better in this respect than simply feeding the rats with other calcium compounds or whether it is better than any of these compounds injected intravenously.

It is of interest, however, that, according to Shohl [1939], calcium is present in serum in a concentration of only 10·0 to 10·5 mg. per 100 c.c. of serum, and that unless an animal has been on a calcium deficient diet the ingestion of calcium salts produces only a minute rise in serum calcium. This rise is so small that for some time it was thought not to be significant.

On the other hand, Bell, Cuthbertson, and Orr [1941] stated that a daily consumption by growing rats of dietary calcium up to ·36 g. of calcium per 100 g. (dry weight) of food increased the weight, the calcium content, the bending and twisting strengths, and the thickness of the cortex, of their femora. Greater amounts of calcium had no effect. An accurate estimation of the calcium content of the diet fed to the rats used for this work has not been possible, but approximate calculations show that the percentage of calcium in their food approached the amount in the diet used by Bell *et al.*

Rats kept on a basal diet supplemented with orange juice have been shown by Lanford [1939] to absorb more calcium from the food than the control animals on the basal diet alone. The work of Ruskin [1938] suggests that the factor in orange juice which aids calcium

absorption may be vitamin C, although Henry and Kon [1939] found that rats on a diet adequate in phosphorus, but low in calcium, did not store more calcium if 2 mg. of vitamin C were added daily to the basal diet. Ruskin, however, appears to believe that the formation of calcium ascorbate in the small intestine is a preliminary to the absorption of calcium. He has pointed out that vitamin C dissolves calcium carbonate with a rapidity second only to fairly strong HCl. Ca ascorbate itself is a yellowish white amorphous powder which, according to Ruskin, is hygroscopic. One important point about calcium ascorbate is that the double bond structure of ascorbic acid is unaffected by the attachment of calcium to the vitamin C molecule, and the vitamin therefore retains its reducing power, and hence its biological activity.

Calcium ascorbate has a greater solubility and possibly a greater degree of ionisation than even the soluble glucono-galacto-gluconate of calcium. Therefore the present results may be due in part to the greater ease with which the calcium is absorbed from the subcutaneous tissues when the ascorbate is injected.

The availability of calcium to the body appears to some extent to be dependent upon how much of it is attached to serum proteins. Blood serum contains about three times as much calcium as can be held in an inorganic solution, due to the fact that an appreciable portion of the calcium is attached to the serum proteins. Ruskin and Jonnard [1938] showed, by means of a refractometer, that if calcium is added to blood as the ascorbate, the proteins fixed 0·8 mg. per c.c. On the other hand, if the calcium is added as calcium chloride, only about 0·4 to 0·5 mg. per c.c. of calcium is fixed by the proteins. If calcium gluconate is used, only 0·2 mg. is fixed.

The evidence available suggests, therefore, that Ca ascorbate is better ionised, more easily absorbed, and results in a higher concentration of calcium in the blood than other salts of calcium. This indicates that it may be an ideal form for therapeutic injection of calcium salts. Support for this contention is given by Ruskin, who claims that it is non-irritating by injection whether administered subcutaneously, intramuscularly, or intravenously, and that it is well tolerated by the gastro-intestinal tract. The same cannot be said of other calcium salts. For example, Aub [1937] points out that two salts of calcium may be used for injection—calcium chloride and calcium gluconate. The use of intravenous calcium chloride has numerous disadvantages, among which may be an occasional thrombosis of a vein, or, if some of it happens to be injected extravascularly, great pain and sloughing of the affected area are likely to result. Even intramuscular injections of calcium gluconate may cause vomiting in the patient, apart from what is described as the characteristic calcium effect of a feeling of great heat suffusing through the body. Aub points out that calcium salts

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should always be injected very slowly, preferably over a period of not less than 5 minutes.

The amount of calcium injected into rats as ascorbate was equal to 5 mg. of Ca, and this was the equivalent of 3·5 g. of calcium injected into a 70-kilogram man. McCance and Widdowson [1939], however, found that the injection 0·186 g. of calcium intravenously was an unconfortable experience, as it was accompanied by constant flushings and nausea. However, these authors injected the calcium as the gluconate, and it may prove possible to inject larger amounts of calcium with fewer unpleasant results if the ascorbate is used. This is a matter, however, for further investigation.

Summary.

A new technique has been described for the estimation of the power of a substance to accelerate the healing of bone.

It has been found by this method that neither vitamin C nor calcium glucono-galacto-gluconate in the doses given accelerates the healing of bone in rats on an adequate diet when injected subcutaneously.

On the other hand, it has been found that calcium ascorbate injected subcutaneously into rats does statistically increase the amount of bone regenerated by the end of 7 days.

The vitamin C used for these experiments was Redoxon, Roche, and I am greatly indebted to Roche Products Ltd., not only for the vitamin C, but also for making the calcium ascorbate specially for this work at my request.

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pages 567 - 570

Ascorp. # 253

Physiology - New characteristics of the protection exerted by ascorbic acid against B1 deficiency. Note (*) by Mlle. Therese Terroine (1) presented by Maurice Lemoigne.

Ascorbic acid considerably lowers the creatinuria of B1 deficiency in the Rat; it easily lowers the hypercreatininutia of the young subject but is without effect on the deficient adult. Methylene blue, thionine, succinic acid, $ClNH_4$ have no "vicariant" activity in B1 deficiency. This activity is exerted by isoascorbic activity with the same intensity as by ascorbic acid.

In addition to those previously uncovered (2) there are two new characteristics of the "vicariant" role of ascorbic acid B1 avitaminosis which will be analyzed successively.

1. Partial inhibition of hypercreatinuria and creatininuria of B1 deficiency, by ascorbic acid. - Male albino rats, young, weighing 60 g initially, and adult, are divided into four lots, subjected respectively, to one of the following diets, the composition of which has already been given (2) : ordinary B1 deficient diet, the preceding diet plus 3% ascorbic acid, complete control diet given in limited quantity, equal to that ingested by the B1 deficient lot, not supplemented with ascorbic acid, and control diet given ad libitum.

After 35 hours of treatment, the urinary measurements of the creatinic bodies are made by the Folin method. From the collection of our results, the essentials of which appear in the table below, it appears that:

Column I Nature of diet
II Weight of animals
III Ingested (g per day)
IV Urinary creatin (mg/100g/24 hr)
V Urinary Creatinin (mg/100g/24hr)

I	II	III	IV	V
1. Young rats				
Control, ad lib	193 (150-224)	18	0.77 (0.57-1.09)	4.97 (4.56-5.65)
Control, limited	107 (87-120)	5	1.75 (0.89-3.34)	4.70 (3.54-5.61)
B1 deficient	94 (88-104)	5	7.21 (4.02-9.76)	8.38 (7.08-10.0)
B1 deficient + 3% ascorbic acid	160 (136-188)	14	1.61 (1.19-2.03)	6.63 (5.33-8.84)
2. Adult rats				
Control, ad lib	288 (267-301)	18	0.64 (0.55-0.71)	5.40 (5.41-5.86)
Control, limited	223 (218-228)		0.50 (0.25-0.78)	5.53 (4.78-5.93)
B1 deficient	197 (189-207)	5 5	3.54 (2.02-5.66)	7.14 (6.20-8.48)
B1 deficient + 3% ascorbic acid	269 (252-277)	17	1.04 (0.97-1.13)	6.88 (6.62-7.27)

1. Ascorbic acid vigorously inhibits, without completely eradicating, the very high creatinuria the B1 deficient young rat or adult rat. This hypercreatinuria is very characteristic, although not specific, of B1 avitaminosis, because we were careful to record it six days before the death; it cannot therefore be attributed to the troubled nitrogen metabolism noted in the immediate pre-mortal phase. The animals, furthermore, during the measurements, show none of the nervous symptoms characteristic of the ultimate phase of B1 deficiency.

2. The considerable drop in the creatinuria of B1 deficiency owed to ascorbic acid occurred in spite of the fact that the vitamin B1 exhaustion of the liver and the muscle was identical to, or slightly less than, that observed in the deficient animals which were not supplemented (see table).

3. Ascorbic acid, on the other hand, is not very capable, in the young rat, and totally incapable in the adult, to reduce the hypercreatininuria of B1 deficiency.

	(Concentration?) in vitamin B1 -- g/g fresh)		
	Liver		Muscle
Nature of diet	Young rats	Adult rats	(Young ?) rats
Control, limited	9.18 (7.5-10.05)	8.1 (7.2-12.9)	(illegible) (1.4 - 2.3?)
B1 deficient	0.73 (0.25-1.32 ?)	0.50 (0.19-0.87)	0.6 (?) (0.5-0.9)
B1 deficient + 3% ascorbic acid	0.39 (0.20-1.0)	1.10 (0.49-1.?)	0.6 (0.4-0.9)

2. Study of causes of the vicariant role of ascorbic acid in B1 deficiency - Is the favorable influence of ascorbic acid due to the value of its pH (E'_o = 0.058 at pH 7)?, to its acid function ?, to the integrity of constitution of its molecule as a whole ? To examine the value of these hypotheses, we tried to find out whether or not this favorable influence is shared: 1) with agents of oxydo-reduction having an pH close to that of ascorbic acid, such as thionin (E'_o = 0.062)or methylene blue (E'_o = 0.011; 2) with acids such as succinic acid or generators of acid such as $ClNH_4$; 3) with an isomer of ascorbic acid, isoascorbic acid.

See figure, page 569.

(Ordinate and abscissa designations illegible)

Ponderal evolution of rats receiving:

1. A normal diet ad libitum
2. A diet deficient in B1 and supplemented by 3% ascorbic acid
3. A diet deficient in B1 and supplemented by 3% isoascorbic acid,
4. A normal, limited diet

5. A diet deficient in B1

6. A diet deficient in B1 and supplemented with 3% succinic acid

7. Diet deficient in B1 and supplemented with 0.1% $ClNH_4$,

8. Diet deficient in B1 and supplemented with 0.09% thionin

9. Diet deficient in B1 and supplemented with 0.12% methylene blue

---

The consequences of the administration of each of these substances on the graph of the ponderal evolution, in the course of B1 deficiency of separate rats appear in the figure above.

These results lead us to assume that:

1) The causes of the vicariant role of ascorbic acid in B1 deficiency do not arise solely from its level of oxydo-reduction or its acid function, otherwise agents of oxydo-reduction with similar rH or acid agents, would have probably exerted, by analogy, some degree, of protection against B1 avvitaminosis.

2) The vicariant faculty of ascorbic acid is not linked to its quality as "vitamin c." This faculty is, as a matter of fact, shared equally with isoascorbic acid, which differs in constitution, and almost totally lacks antiscorbutic power. The cause of the protective power of ascorbic acid against B1 deficiency still remains to be discovered.

(*) Session of July 22, 1957

(1) With the technical collaboration of Mlles.F. Buisson and G. Roux.

(2) Th. Terroine, Comptes Rendues, 242, 1956, p. 2767, and 243, page 968.

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Ascorb. #253

présynaptique et postsynaptique au niveau de leur apposition. De nombreuses vésicules sont visibles dans les terminaisons nerveuses. (× 25 500.)

Fig. 2. — Ganglion cervical supérieur (Rat). Au sein d'un gliocyte, fibre nerveuse remplie de vésicules au voisinage de la surface d'un neurone, mais sans rapport avec elle. « Mesaxons » reliant la fibre aux membranes externe et interne du gliocyte. (× 27 000.)

Fig. 3. — Ganglion cervical supérieur (Rat). Fibre amyélinique simple attachée par un long « mesaxon » à la surface du gliocyte engaînant. (× 31 000.)

Fig. 4. — Ganglion cervical supérieur (Rat). Fibre nerveuse chargée de vésicules. (× 38 000.)

Fig. 5. — Ganglion cervical supérieur (Rat). Fibre nerveuse chargée de vésicules accolée à un fin prolongement, qui pourrait être dendritique. (× 27 000.)

Fig. 6. — Ganglion cervical supérieur (Rat). Dans la mince couche gliale qui borde le neurone, vers le bas de la figure, fin prolongement séparé du neurone par une épaisseur de glie d'environ un vingtième de micron. Le « mesaxon » émet une très courte branche latérale vers la surface du gliocyte appliquée contre le neurone. Vers le haut de la figure, toujours dans la même couche gliale, et à la même distance du neurone, deux éléments de nature indéterminée, étroitement accolés et liés aux mêmes « mesaxons ». (× 30 000.)

(*) Séance du 22 juillet 1957.

(1) *J. Biophys. Bioch. Cyt.*, 1, 1955, p. 69-88.
(2) *Fed. Proc.*, 13, 1954, p. 35.
(3) *J. Biophys. Bioch. Cyt.*, 1, 1955, p. 47-56.
(4) *Anat. Rec.*, 118, 1954, p. 335.
(5) *Cold. Spring Harb. Symp. Quant. Biol.*, 17, 1952, p. 32-36.

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PHYSIOLOGIE. — *Nouveaux caractères de la protection exercée par l'acide ascorbique contre la carence* B_1. Note (*) de Mlle **Thérèse Terroine** (1), présentée par M. Maurice Lemoigne.

L'acide ascorbique réduit considérablement la créatinurie de carence B_1 chez le Rat; il abaisse faiblement l'hypercréatininurie du jeune mais est sans effet sur celle de l'adulte carencé. Le bleu de méthylène, la thionine, l'acide succinique, le $ClNH_4$ n'ont aucune activité vicariante en carence B_1. Cette activité est exercée par l'acide isoascorbique avec la même intensité que par l'acide ascorbique.

S'ajoutant à ceux antérieurement mis en évidence (2), deux nouveaux caractères du rôle vicariant de l'acide ascorbique en avitaminose B_1 vont être analysés successivement.

1. *Inhibition partielle des hypercréatinurie et créatininurie de carence* B_1 *par l'acide ascorbique.* — Des rats mâles albinos, soit jeunes pesant initialement 60 g, soit adultes, sont divisés en quatre lots soumis respectivement à l'un des régimes suivants dont la composition a été déjà donnée (2) : régime de carence ordinaire en B_1, le régime précédent additionné de 3 % d'acide ascorbique, régime témoin *complet* donné en *quantité restreinte* égale à celle ingérée par le lot carencé en B_1 non supplémenté d'acide ascorbique; régime témoin donné *ad libitum*.

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Après 35 jours de traitement les déterminations urinaires des corps créatiniques sont effectuées par la méthode de Folin. De l'ensemble de nos résultats, dont l'essentiel apparaît dans le tableau suivant, il ressort que :

Nature des régimes.	Poids des animaux.	Ingéré (g par j).	Créatine urinaire (mg/100 g/24 h).	Créatinine urinaire (mg/100 g/24 h).
		1° *Rats jeunes.*		
Régime témoin *ad libitum*...	193 (150–224)	18	0,77 (0,57–1,09)	4,97 (4,56–5,65)
Régime témoin *restreint*...	107 (87–120)	5	1,75 (0,89–3,34)	4,70 (3,54–5,61)
Régime carencé en B_1.....	94 (88–104)	5	7,21 (4,02–9,76)	8,38 (7,08–10,0)
Régime carencé en B_1 + 3 % d'acide ascorbique.....	160 (136–188)	14	1,61 (1,19–2,03)	6,63 (5,33–8,84)
		2° *Rats adultes.*		
Régime témoin *ad libitum*...	288 (267–301)	18	0,64 (0,55–0,71)	5,40 (5,41–5,86)
Régime témoin *restreint*...	223 (218–228)		0,50 (0,35–0,78)	5,53 (4,78–5,93)
Régime carencé en B_1.....	197 (189–207)	5 5	3,54 (2,02–5,66)	7,14 (6,20–8,48)
Régime carencé en B_1 + 3 % d'acide ascorbique.....	269 (252–277)	17	1,04 (0,97–1,13)	6,88 (6,62–7,27)

1° L'acide ascorbique inhibe énergiquement, sans l'enrayer totalement, la très forte créatinurie du Rat jeune ou adulte en carence B_1. Cette hypercréatinurie est bien caractéristique, sinon spécifique, de l'avitaminose B_1, car nous avons pris soin de l'enregistrer six jours au moins avant la mort; elle ne peut donc être imputée au trouble du métabolisme azoté s'observant dans la phase immédiatement prémortelle. Les animaux ne présentent, en outre, durant les déterminations, aucun des symptômes nerveux caractéristiques de la phase ultime de la carence B_1;

2° L'abaissement considérable de la créatinurie de carence B_1 par l'acide ascorbique a lieu bien que l'épuisement en vitamine B_1 du foie et du muscle soit identique ou légèrement inférieur à celui observé chez les animaux carencés non supplémentés (*voir* tableau).

3° L'acide ascorbique est, en revanche, peu apte chez le Rat jeune et totalement inapte chez le Rat adulte à réduire l'hypercréatininurie de carence B_1.

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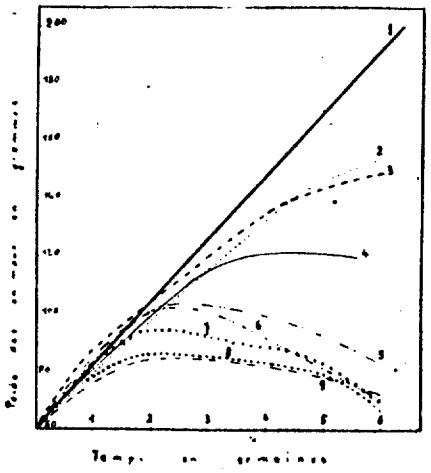
Nature des régimes.	Concentration en vitamine B_1 (γ/g frais). Foie. Rats jeunes.	Foie. Rats adultes.	Muscle. Rats jeunes.
Régime témoin *restreint* ...	9,18 (7,5 – 10,95)	8,1 (7,2 – 12,9)	[illegible] (1,4 – 2,5)
Régime carencé en B_1.....	0,73 (0,25 – 1,32)	0,50 (0,19 – 0,87)	0,6 (0,5 – 0,9)
Régime carencé en B_1 + 3 % d'acide ascorbique.....	0,39 (0,20 – 1,0)	1,10 (0,49 – [illegible])	0,6 (0,4 – 0,9)

2. *Recherche des causes du rôle vicariant de l'acide ascorbique en carence* B_1. — L'influence favorable de l'acide ascorbique est-elle due à la valeur de son *r*H ($E'_0 = 0{,}058$ à pH 7) ? à sa fonction acide ? à l'intégrité de constitution de sa molécule toute entière ? Pour examiner la valeur de ces hypothèses nous avons recherché si cette influence favorable est ou non partagée : 1° avec des agents d'oxydo-réduction ayant un *r*H voisin de celui de l'acide ascorbique tel la thionine ($E'_0 = 0{,}062$) ou le bleu de méthylène ($E'_0 = 0{,}011$); 2° avec des acides tel l'acide succinique ou des générateurs d'acide comme $ClNH_4$; 3° avec un isomère de l'acide ascorbique, l'acide isoascorbique.



Évolution pondérale comparée de rats recevant :

1. un régime normal *ad libitum*;
2. un régime carencé en B_1 et supplémenté de 3 % d'acide ascorbique;
3. un régime carencé en B_1 et supplémenté de 3 % d'acide isoascorbique;
4. un régime *normal limité*;
5. un régime carencé en B_1;
6. un régime carencé en B_1 et supplémenté avec 3 % d'acide succinique;
7. régime carencé en B_1 et supplémenté avec 0,1 % $ClNH_4$;
8. régime carencé en B_1 et supplémenté avec 0,09 % de thionine;
9. régime carencé en B_1 et supplémenté avec 0,12 % de bleu de méthylène.

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Les conséquences de l'administration de chacune de ces substances sur l'allure de l'évolution pondérale tout au long de la carence B_1 de lots distincts de rats apparaissent dans la figure ci-dessus.

Ces résultats conduisent à supposer que :

1° Les causes du rôle vicariant de l'acide ascorbique en carence B_1 ne relèvent pas strictement de son niveau d'oxydo-réduction ou de sa fonction acide sinon des agents d'oxydo-réduction de rH voisin où des agents acides auraient vraisemblablement exercé par analogie, à un degré quelconque, une protection contre l'avitaminose B_1;

2° La faculté vicariante de l'acide ascorbique n'est pas liée à sa qualité de « vitamine *c* ». Cette faculté est, en effet, partagée à égalité avec l'acide isoascorbique, de constitution différente, et presque totalement dénué de pouvoir antiscorbutique. La cause du pouvoir protecteur de l'acide ascorbique contre la carence B_1 reste donc entièrement à découvrir.

(*) Séance du 22 juillet 1957.

([1]) Avec la collaboration technique de Mlles F. Buisson et G. Roux.

([2]) Th. Terroine, *Comptes rendus*, **242**, 1956, p. 2767 et **243**, 1956, p. 968.

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NEUROPHYSIOLOGIE. — *Développement du potentiel post-synaptique en présence du potentiel d'action dans le soma neuronique du ganglion d'Escargot* (Helix pomatia). Note (*) de M. **Ladislav Tauc**, présentée par M. Robert Courrier.

Nous avons appliqué la technique d'enregistrement intracellulaire au soma neuronique du ganglion sous-œsophagien de *Helix pomatia*, stimulé par voie orthodromique, c'est-à-dire par l'intermédiaire des jonctions synaptiques (schéma, *fig.* 1). Étant donné la multiplicité des voies afférentes qui aboutissent à la cellule, on peut graduer l'intensité de cette excitation indirecte en augmentant par l'intensité du stimulus le nombre de fibres activées. Dans ces conditions, on fait apparaître d'abord une onde lente, le potentiel post-synaptique d'excitation (*fig.* 1, tracé 5), qui engendre lui-même un (ou plusieurs) potentiel d'action (*fig.* 1, tracés 1, 2, 3, 4). En 6 de la figure 1, nous montrons le potentiel d'action isolé obtenu par stimulation électrique directe de la cellule par une deuxième électrode intracellulaire.

Dans la présente Note, nous nous sommes intéressé à la répercussion possible du potentiel d'action sur le cours du potentiel post-synaptique. Étant donné que le processus tout ou rien représente un bouleversement membranaire important, il peut sembler que le processus post-synaptique soit condamné à s'effacer après l'apparition du potentiel d'action. Mais

SUMMARY

1. Muscle extracts of twenty different species of fish have been examined electrophoretically at 0·05*I*, pH 7·5.

2. Each species has a characteristic electrophoretic diagram, and considerable differences in mobility and composition exist between species.

3. The diagrams may, however, be still divided into definite mobility groups of approximately consistent quantitative size.

4. The diagrams of related species appear to be as dissimilar as those of unrelated species.

5. The effect of age and of species variants on the diagrams has been examined.

6. Elasmobranch muscle appears to contain proteins with unusually high isoelectric points.

7. The question of the identity of components of different diagrams has been discussed. Whatever the answer, it is certain that components which are present in some species are completely absent in others.

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99

Comparative Observations on the Chemical Properties and Biological Activities of L-Ascorbic and D-*iso*Ascorbic Acids

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In a previous publication the course of oxidation of L-ascorbic acid under various conditions and in the presence of different accompanying substances was described (Leibowitz & Guggenheim, 1938*a*). It was shown that the stability of the vitamin in solution depends specifically on the nature of the accompanying substances and on the pH. It has also been shown that L-ascorbic acid exerts a detoxifying action on potassium cyanide, phenol and tetanus toxin (Leibowitz & Guggenheim, 1938*b*; Kligler, Guggenheim & Warburg, 1938). Some evidence, derived from titrimetric and cryoscopic data, indicated that the detoxifying effect results from the reversible formation of a chemical compound between the vitamin and potassium cyanide or phenol. Other authors have reported that ascorbic acid diminishes the toxicity of various other substances such as arsphenamines, lead, sulphonamides, benzene, trinitrotoluene, and anaesthetics (Bicknell & Prescott, 1948). It has been tentatively suggested that the antiscorbutic function of L-ascorbic acid may be at least partially due to its ability to detoxify naturally produced toxic substances (Leibowitz & Guggenheim, 1938*b*).

Numerous clinical and experimental observations have indicated that ascorbic acid plays a role in certain forms of anaemia (Vilter, 1947; Dyke, Della Vida & Delikat, 1942; May, Nelson, Lowe & Salmon, 1950). Studies by Nichol & Welch (1950) and Welch, Nichol, Anker & Boehne (1951) have

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shown that ascorbic acid promotes the formation of citrovorum factor from folic acid.

A series of chemical analogues of L-ascorbic acid has been prepared synthetically (Smith, 1946). One of these compounds, *iso*ascorbic acid (D-araboascorbic acid) shows great chemical similarity to vitamin C. This compound, however, has only one-twentieth of the antiscorbutic activity of L-ascorbic acid (Dalmer & Moll, 1933; Demole, 1934). These facts afforded the opportunity of testing our hypothesis concerning the relationship between the antiscorbutic activity of vitamin C and the above-mentioned properties. The two compounds were compared as to: (1) their ability to form complexes with potassium cyanide or phenol; (2) their detoxifying effect on potassium cyanide, phenol and tetanus toxin; and (3) their ability to increase urinary excretion of citrovorum factor after administration of folic acid.

MATERIALS AND METHODS

The ability of ascorbic acid and of *iso*ascorbic acid to detoxify potassium cyanide was tested with *Escherichia coli*. The bacteria were grown in nutrient broth, and the amount of growth obtained after 24 hr. was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter. Tetanus toxin was prepared in the following way: *Clostridium tetani* was grown in Tarozzi broth at 37°. After 10 days broth was filtered and centrifuged, and the toxin-containing supernatant was tested on young mice weighing 15–25 g. Appropriately diluted broth was injected intramuscularly allowing 0·1 ml./10 g. of body weight. The mice were observed for 6 days and the mortality from tetanus noted.

The conversion of folic acid into citrovorum factor was studied with six adult rats weighing 200–300 g. They received a basal diet consisting of corn starch 73, casein 18, olive oil 5, and salt mixture 4%, which was supplemented with 0·3 mg. riboflavin, 0·2 mg. thiamine, 0·1 mg. pyridoxine, 1·6 mg. calcium pantothenate, and 100 mg. choline chloride per 100 g. ration. Each rat received 100 i.u. vitamin A and 4 i.u. vitamin D twice weekly. Citrovorum factor was determined with *Leuconostoc citrovorum* (ATCC 8081) according to Sauberlich & Baumann (1948). After incubation for 3 days at 37° the acid produced was titrated with 0·02N-NaOH. Synthetic citrovorum factor (leucovorin)* in the form of the hydrated barium salt was used as standard.

RESULTS

Polarimetric studies

Experiments on the change in the rotation values of ascorbic acid and *iso*ascorbic acid induced by potassium cyanide will be described elsewhere. These experiments require special treatment because of the complications arising from the powerful effect of pH on the optical activities of both acids. Data typical of many experiments with phenol are presented in Table 1. Small but significant changes are induced in the optical rotations of both ascorbic acid and *iso*ascorbic acid, the dextrorotation of the former being slightly decreased and the laevorotation of the latter slightly increased. The order of magnitude of the effects is almost the same in both cases. The effects are immediate but are somewhat intensified by incubation at 30°.

Table 1. *Effect of phenol on the optical rotation of ascorbic and* iso*ascorbic acids in aqueous solutions*

[Ascorbic acid] (M)	[*iso*Ascorbic acid] (M)	[Phenol] (M)	Incubation at 30° (hr.)	α_D (1 dm.) (°)
—	—	—	0	+0·39
0·1	—	—	6	+0·38
0·1	—	0·1	0	+0·36
0·1	—	0·1	6	+0·36
0·1	—	0·1	24	+0·34
0·4	—	—	0	+1·79
0·4	—	—	6	+1·77
0·4	—	—	24	+1·78
0·4	—	0·1	0	+1·74
0·4	—	0·4	0	+1·70
0·4	—	0·4	6	+1·71
0·4	—	0·4	24	+1·70
—	0·1	—	0	−0·28
—	0·1	—	6	−0·26
—	0·1	0·1	0	−0·30
—	0·1	0·1	6	−0·30
—	0·1	0·1	24	−0·31
—	0·4	—	0	−1·09
—	0·4	0·4	0	−1·15
—	0·4	0·4	6	−1·18
—	0·4	0·4	24	−1·17

Cryoscopic studies

The freezing-point depressions resulting from the mixing of equal amounts of equimolecular aqueous solutions of ascorbic acid or *iso*ascorbic acid with potassium cyanide or phenol were measured. The freezing-point depressions of the mixtures were measured immediately after mixing. The half-values of the sums of the depressions of the components were taken as 'calculated' values (Table 2).

The experimental values of the freezing-point depressions of mixtures of each of the acids with potassium cyanide or phenol were appreciably less than those calculated from separate depressions of the components, indicating a decrease in molecular concentration, due to partial combination of the acids with the reagents to form condensation or addition products. The effect was more pronounced with potassium cyanide than with phenol. No further change in the depression values was induced by incubation of the mixtures at room temperature or at 30° for 24 hr.

* Leucovorin was supplied by the Lederle Laboratories Division, American Cyanamid Company, by the courtesy of Dr T. H. Jukes.

Table 2. *Effect of potassium cyanide and phenol on cryoscopic determinations in solutions of ascorbic and* isoascorbic *acids*

[Ascorbic acid] (M)	[*iso*Ascorbic acid] (M)	[KCN] (M)	[Phenol] (M)	Δ_{Found} (°)	$\Delta_{Calculated}$ (°)
0·1	—	—	—	0·203	—
—	0·1	—	—	0·210	—
—	—	0·1	—	0·354	—
0·05	—	0·05	—	0·257	0·278
—	0·05	0·05	—	0·262	0·282
0·2	—	—	—	0·402	—
—	0·2	—	—	0·412	—
—	—	—	0·2	0·389	—
0·1	—	—	0·1	0·385	0·396
—	0·1	—	0·1	0·393	0·401

Table 3. *Effect of ascorbic and* isoascorbic *acids on the potassium cyanide inhibition of* Esch. coli

(The figures indicate galvanometer readings obtained after 24 hr. incubation of bacteria with solutions of KCN, ascorbic acid and *iso*ascorbic acid. Readings adjusted to 200 for culture without substances added. Average of three sets of experiments.)

[KCN] (M)	[Ascorbic acid] (M)	[*iso*Ascorbic acid] (M)	Substances: Freshly dissolved	Dissolved and mixed together 24 hr. before the onset of incubation	Dissolved and kept separately for 24 hr. before the onset of incubation
—	—	—	200	200	200
—	0·01	—	258	254	255
—	—	0·01	243	212	222
0·02	—	—	0	25	25
0·01	—	—	54	81	81
0·005	—	—	71	102	102
0·02	0·01	—	0	141	54
0·01	0·01	—	51	176	118
0·005	0·01	—	77	192	113
0·02	—	0·01	10	91	23
0·01	—	0·01	33	139	103
0·005	—	0·01	93	155	125

Detoxication of potassium cyanide

Three experiments were performed. In the first experiment neutralized solutions of potassium cyanide, ascorbic acid and *iso*ascorbic acid were added to samples of nutrient broth in amounts to give the final concentration indicated in Table 3. Sufficient water was added to bring the volume to 5 ml.; then the test tubes were inoculated with *Esch. coli*. In the second experiment similar mixtures of potassium cyanide, ascorbic acid and *iso*ascorbic acid solutions were incubated at room temperature for 24 hr. before being added to the nutrient broth. In the third experiment which served as control for the second, the solutions of potassium cyanide, ascorbic acid and *iso*ascorbic acid were kept separately at room temperature for 24 hr. and separately added to the nutrient broth immediately before inoculation. The amount of growth obtained after 24 hr. incubation is shown in Table 3.

It may be observed that neither ascorbic acid nor *iso*ascorbic acid was able to affect the potassium cyanide inhibition of growth of *Esch. coli* when added separately immediately prior to incubation. If, however, the ascorbic acid or *iso*ascorbic acid were pre-incubated with potassium cyanide for 24 hr., the mixtures were far less toxic for the bacteria than the same concentrations of potassium cyanide alone. These findings confirm previous results (Leibowitz & Guggenheim, 1938*b*) obtained with ascorbic acid. It is noteworthy that no marked difference was noted between the detoxifying effect of ascorbic acid and *iso*ascorbic acid.

Detoxication of phenol

Solutions of phenol (50 mg./ml.) were incubated with equimolar quantities of ascorbic acid or *iso*ascorbic acid at 40° for 4 hr. The toxicity of these solutions was tested on young rats weighing 50–100 g.; the dose injected subcutaneously contained 50 mg. phenol per 100 g. of body weight. Three groups, each comprising twenty-two animals were used: the first group received phenol-ascorbic acid, the second phenol-*iso*ascorbic acid, the third phenol alone. In the first group six rats died, in the

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second twelve, whereas in the third group all twenty-two animals succumbed. The difference in mortality between the first or the second group and the third group proved to be statistically significant, whereas the difference between the first and the second group was insignificant. Ascorbic acid and *iso*ascorbic acid are, therefore, equally active in detoxifying phenol.

Detoxication of tetanus toxin

Equal volumes of the toxic broth and of solutions of ascorbic acid or *iso*ascorbic acid (1 mg./ml.) were incubated at 37° for 24 hr. In the first experiment the broth was diluted 1:100 and injected into ten mice. All these mice died during the next 6 days. Control groups given the toxin-ascorbic acid or the toxin-*iso*ascorbic acid mixture suffered no loss during the same time. In the second experiment two groups of thirty mice were injected with a solution diluted 1:8 of the toxic broth which had been incubated with ascorbic acid or *iso*ascorbic acid. The mortality during the following 6 days amounted to twenty and twenty-one mice, respectively. Thus no difference was noted in the ability of ascorbic acid and *iso*ascorbic acid to detoxify tetanus toxin.

Conversion of folic acid to citrovorum factor

Rats were maintained for 6 days on the normal diet. During the following 6 days the rats received 1 mg. folic acid daily intraperitoneally. During the third and fourth 6-day periods the rats received daily injections of folic acid and 17·6 mg. of ascorbic acid or *iso*ascorbic acid. Urine was collected and analysed for citrovorum factor during the last 3 days of each period. Table 4 shows the average daily excretion of citrovorum factor during these four periods.

Table 4. *Urinary excretion of citrovorum factor by rats*

(Average of six rats; means and standard errors.)

Substances injected	Citrovorum factor in urine (μg./day/rat)
None	2·9 ± 0·32
Folic acid	13·5 ± 5·38
Folic acid + ascorbic acid	142·0 ± 31·25
Folic acid + *iso*ascorbic acid	153·0 ± 14·58

It follows from Table 4 that both ascorbic acid and *iso*ascorbic acid increase urinary excretion of citrovorum factor. *iso*Ascorbic acid was found to be as active in this respect as ascorbic acid.

DISCUSSION

The similarity which exists between the chemical properties of ascorbic acid and *iso*ascorbic acid extends to their biological activities as well. The two compounds have almost the same ability to combine with potassium cyanide or phenol as measured polarimetrically or cryoscopically. Moreover, both ascorbic acid and *iso*ascorbic acid prevent the toxic effects of potassium cyanide, phenol and tetanus toxin on *Esch. coli*, rats, and mice, respectively. Ascorbic acid and *iso*ascorbic acid also show practically the same effect on the conversion of folic acid into citrovorum factor in rats. Identical actions of ascorbic acid and its analogues on other biological systems have already been described. L- and D-Ascorbic acids are equally effective in increasing the height of contraction of the isolated frog heart (Krayer, Linstead & Todd, 1943) and in reducing the toxicity of neoarsphenamine (McChesney, Barlow & Klinck, 1944). Ascorbic acid and *iso*ascorbic acid exert the same effect in promoting the oxidation of *p*-hydroxyphenylpyruvic acid (Knox & LeMay-Knox, 1951) and of tyrosine by liver homogenates (Sealock, Goodland, Summerwell & Brierly, 1952). *iso*Ascorbic acid can replace ascorbic acid as growth factor for *Trichomonas* (Cailleau, 1939) but not for trypanosomes (Lwoff, 1951).

The role played by ascorbic acid in the metabolic activation of folic acid has been studied by Welch and co-workers (Nichol & Welch, 1950; Welch *et al.* 1951). They found that the addition of either ascorbic acid or its analogue glucoascorbic acid to liver slices *in vitro* markedly increases the conversion of folic acid to citrovorum factor, and that both substances increase the excretion of citrovorum factor when administered together with folic acid. On the other hand, such potent reducing agents as cysteine and glutathione had little or no influence.

Our experiments show that *iso*ascorbic acid shares with ascorbic acid the ability to detoxify potassium cyanide and phenol and to stimulate the conversion of folic acid to citrovorum factor in the rats. Since the antiscorbutic activity of *iso*ascorbic acid is negligible, it seems impossible to assume that the antiscorbutic activity of ascorbic acid is due to the above-mentioned chemical and biological properties.

SUMMARY

1. Ascorbic and *iso*ascorbic acids (D-araboascorbic acid) show similar abilities to combine with potassium cyanide and phenol in aqueous solutions.

2. Ascorbic and *iso*ascorbic acids are equally effective in detoxifying potassium cyanide, phenol and tetanus toxin, and in the conversion of folic acid into citrovorum factor.

3. The antiscorbutic activity of ascorbic acid cannot be explained on the basis of the detoxifying activity of this substance.

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The Effect of L-Ascorbic Acid on the *in vitro* Activity of Polyphenoloxidase from Potato

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In recent years many studies of the action of polyphenoloxidase on various substrates, *in vitro*, have been carried out in the presence of L-ascorbic acid (Asimov & Dawson, 1950; Miller, Mallette, Roth & Dawson, 1944; Robinson & Nelson, 1944; Sreerangachar, 1943). In these studies the L-ascorbic acid has been assigned the role of a reducing agent for any *o*-quinones produced during the oxidation of the substrates. It has been generally assumed (Asimov & Dawson, 1950; Miller & Dawson, 1941; Kendal, 1949; Sreerangachar, 1943) that the ascorbic acid has no other effect on the system, although many observers have reported that the rate of oxidation was sometimes significantly different in the presence of ascorbic acid than in its absence (Kendal, 1949; Roberts & Wood, 1950; Krueger, 1950).

Krueger (1950) made a study of the effect of ascorbic acid on the rate of oxidation of tyrosine by mushroom polyphenoloxidase and concluded that initially the ascorbic acid had a 'pro-oxidant' effect on the system since the initial oxygen uptake was substantially increased and the induction period was decreased (cf. Kendal, 1949), but later in the reaction it had an antioxidant effect since the rate of production of L-β-3:4-dihydroxyphenylalanine (dopa) was decreased below that for the ascorbic-acid-free system.

The investigations of Kertesz (1951, 1952) have shown that the observed oxidation rates of polyphenoloxidase systems in the presence of ascorbic acid are markedly dependent on the concentration of cupric ion, and since Krueger apparently did not use copper-free water for his experiments, it seemed worth while to re-investigate this system.

Our experiments, using a colorimetric procedure to determine enzyme activity, show that ascorbic acid has an inhibitory action on potato polyphenoloxidase which is not elicited in the presence of ionic copper. However, when the enzyme was treated with ascorbic acid in the absence of oxygen and the ascorbic acid subsequently removed by dialysis, it was irreversibly changed so that reactivation with cupric salts was not possible. Oxidation products of ascorbic acid, including dehydroascorbic acid, were found to have little effect on the enzyme.

It is suggested that the ascorbic acid affects specific structures of the enzyme (cf. Kertesz, 1951, 1952) in which copper is involved.

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Some Inhibitors of the Fibrinogen-Fibrin Conversion

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According to current concepts the conversion of fibrinogen to fibrin occurs in 3 steps:
Step I (Enzymatic phase):

$$\text{Fibrinogen} \underset{}{\overset{\text{Thrombin}}{\rightleftharpoons}} \text{Fibrin monomer (f)} + \text{fibrino peptides.}$$

Step II (Formation of intermediate polymers):

$$\text{n Fibrin monomer} \rightleftharpoons \text{Intermediate polymer } (f)_n.$$

Step III (Clotting):

$$m\,(f)_n \longrightarrow \text{Fibrin (clot).}$$

The soluble fibrin thus formed may further be converted into insoluble fibrin by the fibrin stabilising factor in presence of calcium ions; but this step is not operative when purified fibrinogen and thrombin are used.

A large number of inhibitors of the fibrinogen-thrombin reaction have been studied during the last 2 decades (Ferry and Shulman, 1949; Shulman, 1951; Edsall and Lever, 1951; Shulman, Katz and Ferry, 1953; Bithel, 1964). Among these inhibitors are polymethylene glycols, neutral amides, guanidine, arginine, cysteine, imidazole, sodium ascorbate, sodium sulphite and EDTA**). Shulman (1951) found that the inhibitors are effective even when added after thrombin at any time before visible clotting is observed; interference with the enzymatic phase was therefore regarded as unlikely. Bithel (1964) too found that EDTA which delayed the fibrinogen-thrombin reaction did not destroy thrombin and hence discounted the possibility of inhibition of the enzymatic phase. It would therefore appear from previous works that interference with the polymerisation phase would be the most probable mode of action of the inhibitors though no direct proof seems to have been put forward. The possibility of an alteration of fibrinogen which would have the same effect on the fibrinogen-thrombin reaction as the inactivation of thrombin (Bithel 1964) has not been systematically investigated. The present work is an attempt at a direct approach to these aspects.

Materials and Methods

All reagents were made in all glass distilled water. In practically all experiments dog plasma was used as the source of fibrinogen and thrombin. Sodium oxalate was used as the anticoagulant.

Fibrinogen: Prothrombin free plasma was obtained by adsorption with $BaSO_4$ (1 g/10 ml plasma) for 3 hrs. This plasma was diluted with 4 vol. of water and the fibrinogen was precipitated by half

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**) Abbreviations: EDTA, ethylene diamine tetra acetic acid; BAL, 2, 3-dimercapto propanol; GSH, glutathione; Tris, tris (Hydroxymethyl) amino methane; TAME, p-toluene sulphonyl arginine methyl ester.

saturation with sodium chloride at 5–10° C. The fibrinogen was collected by centrifugation, washed twice with cold, half-saturated sodium chloride solution, and then dissolved in 0.9% sodium chloride solution. It was purified by two more reprecipitations and finally dissolved in 0.9% NaCl soln.

Thrombin was prepared by Eagle's method as described by Hawk, Oser and Summerson (1954). Since the thrombin contained 5 mM $CaCl_2$, one tenth vol. of 60 mM sodium oxalate was added to the thrombin preparation to precipitate all the calcium and to ensure a slight excess of oxalate. The precipitated calcium oxalate was removed by centrifugation, and the thrombin was stored frozen. It was diluted suitably before use so as to give a thrombin time of 20–30 sec under the experimental conditions in Table 1.

Fibrin monomer was prepared according to the method of Donnelly, Laskowski Jr, Notley, and Scheraga (1955) with the following modifications. A more dilute fibrinogen solution (25 mg/100 ml) was allowed to clot very slowly by the addition of a weak thrombin solution. The clot was collected on a glass rod, washed twice with 0.9% NaCl and dissolved in 1 M NaBr, pH 5.3 (sodium bromide dissolved in 0.05 M acetate buffer, pH 5.3). Appropriate dilutions of fibrin monomer solutions were made with the same solvent.

Temperature: The tests were carried out at room temperature (27°–29° C) unless otherwise stated.

Ionic concentration: The ionic concentration in any set of 'test' and 'control' experiments was kept uniform by the addition of the requisite amount of sodium chloride. As far as possible the total ionic concentration was maintained at or near 300 mEq/l.

Protein estimations were carried out by the method of Daughaday, Lowry, Rosebrough & Fields (1952). Fibrin clots were dissolved in 0.5 ml of warm 0.75 N Na OH, then the alkaline copper tartrate reagent was added and the excess alkali used to dissolve fibrin was neutralised with 0.5 ml of 0.75 N HCl. The subsequent steps of the method remained unchanged.

Thrombin time: The time interval between the addition of thrombin to a fibrinogen solution and the gel point was taken as the thrombin time. The gel point rather than the appearance of fibrin shreds was taken as the index of clotting since in the presence of certain inhibitors the clot remained very clear without the separation of fibrin shreds even long after the gel point.

Esterase activity of thrombin was measured as described by Seegers (1962) using TAME as the substrate.

Effect of inhibitors on the polymerisation of fibrin monomer: Fibrin monomer in 1 M NaBr, pH 5.3 was made to polymerise by dilution with a clotting buffer (0.05 M phosphate buffer pH 6.8) which provides a suitable pH and ionic strength.

The inhibitors under investigation were included in the reaction mixture during polymerisation. In control experiments the inhibitors were added immediately after formaldehyde which was used to stop polymerisation. The fibrin clot formed in the 2 sets of experiments was then estimated.

Opacity of fibrin clot: The polymerisation of fibrin monomer is accompanied by a rise in the opacity. The rate of opacification was measured using an EEL photoelectric colorimeter in 10 ml cylindrical glass cuvettes having a 1 cm light path, with a blue filter (420 mμ). At this wave length, extinction is proportional to light scatter, absorption by proteins being negligible (Ferry & Morrison, 1947). The effect of inhibitors of clotting on polymerisation was studied using opacification as an index of the process.

Results

1. *Effect of Inhibitors on the Thrombin Time of Fibrinogen*

It can be seen from Table 1 that a number of substances notably thiols and EDTA and less effectively sodium sulphite, arginine, sodium ascorbate, hydroxylamine and imidazole are inhibitory. EDTA is effective even at very low concentrations. With progressive dilutions of thrombin the effect of a given inhibitor becomes increasingly manifest although the control values also tend to increase (Fig. 1).

The maximum effect of a given inhibitor is observed at about pH 7.4 which is also the pH at which the thrombin time is minimum (Fig. 2). Similar observations regard-

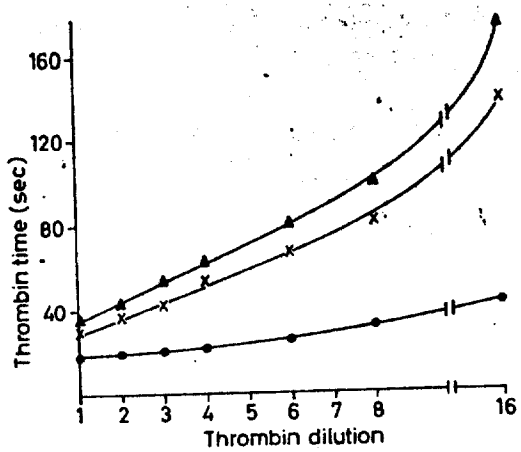

Fig. 1. Effect of thrombin dilution on inhibition of the fibrinogen-thrombin reaction. Experimental conditions same as in exp.3 (b). of Table 1; serial dilutions of thrombin as indicated; concentration of inhibitor 10 mM. ▲——▲ β-mercaptoethanolamine; ×——× Sodium thioglycollate; ●——● Control.

ing the effect of pH and thrombin dilution have been made by Bithel (1964) in regard to EDTA. *Effect of calcium and magnesium ions:* Calcium and to a smaller extent, magnesium ions reduce the effect of inhibitors on the thrombin time of fibrinogen at an optimum concentration of 0.5 to 1 mM (Fig. 3). At higher concentrations (eg. 5 mM) magnesium ions prove to be inhibitory.

From the foregoing it is to be expected that in the presence of calcium ions and using fairly strong thrombin, the effect of inhibitors would be minimal. On the contrary the maximum effect could be demonstrated using calcium free dilute thrombin.

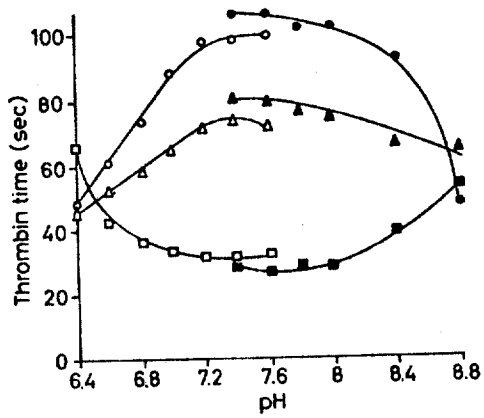

Fig. 2. Effect of pH on the inhibition of fibrinogen-thrombin reaction: Experimental conditions same as in Fig. 1 except that buffers of different pH have been used; strength of thrombin adjusted to give a thrombin time of about 30 sec at pH 7.4. Final concentration of inhibitor 10 mM. ○——○ β-mercaptoethanolamine; △——△ Sodium thioglycollate; □——□ Control; Open characters = Phosphate buffer; Closed characters = Tris buffer.

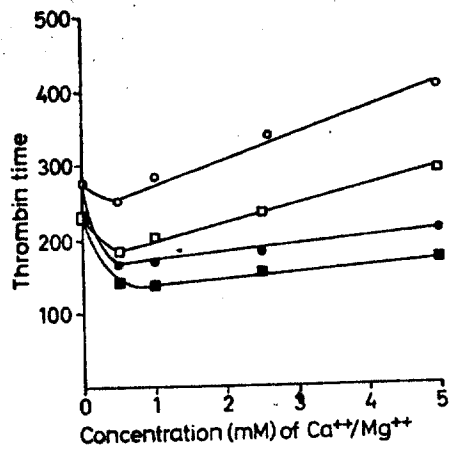

Fig. 3. Effect of Calcium and Magnesium ions on inhibition of fibrinogen-thrombin reaction. Experimental conditions same as in Fig. 2. Final concentration of inhibitor 10mM. Thrombin time expressed as per centage of control value in the absence of inhibitor (25 sec). pH 7.4 Tris buffer. ○——○ β-mercaptoethanolamine + Mg^{++}; □——□ GSH + Mg^{++}; ●——● β-mercaptoethanolamine + Ca^{++}; ■——■ GSH + Ca^{++}.

1. *Effect of Preincubation of Fibrinogen with Inhibitors*

Preincubation of fibrinogen with inhibitors increases their effect on the thrombin time in the case of EDTA (as already reported by Bithel, 1964), sodium sulphite and thioglycollate (Fig.4). Such an effect is not observed with other thiols or arginine.

In view of the above observations it appeared interesting to know whether calcium ions present during preincubation of fibrinogen with inhibitors could counteract the effect of the latter. However, no appreciable difference in thrombin time could be observed no matter whether calcium ions were added along with the inhibitor during preincubation or only along with the thrombin. Calcium ions therefore do not appear to neutralise any effect the inhibitors may have on fibrinogen.

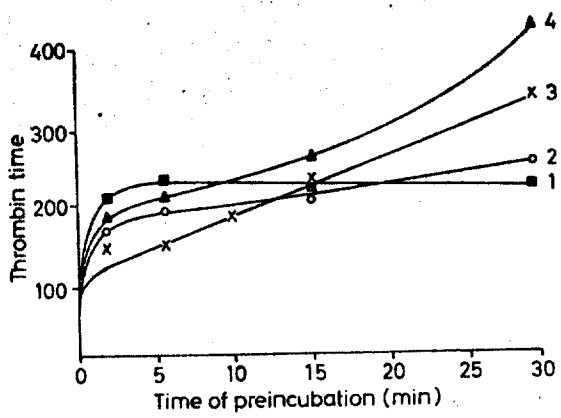

Fig. 4. Effect of preincubation of fibrinogen with inhibitor. Experimental conditions same as in Fig. 2; time of preincubation of fibrinogen with inhibitor as indicated. Thrombin time as percentage of control value in the absence of inhibitor (28 sec). pH 7.4 Tris buffer. 1. BAL 10 mM; 2. Sodium thioglycollate 10 mM; 3. Sodium sulphite 10 mM; 4. EDTA 0.05 mM.

Table 1. Effect of Inhibitors on the Thrombin Time of Fibrinogen.

Expt.	Inhibitor	Additions	Thrombin time (sec) Without inhibitor	With inhibitor
1.	Cysteine	None	26	70
	Cysteine	Ca^{++}	25	42
	Cysteine	Mg^{++}	26	51
	BAL	None	26	67
	Arginine	None	26	39
	Sodium sulphite	None	26	36
	EDTA	None	26	43
	Sodium ascorbate	None	26	34
	Hydroxylamine	None	26	32
	Imidazole	None	26	34
	Guanidine	None	26	33
2.	BAL	None	21	47
	Sodium thioglycollate	None	21	45
3. (a)	Sodium thioglycollate	None	15	16
	β-mercaptoethanolamine	None	15	18
(b)	β-mercaptoethanolamine	None	20	29
	Sodium thioglycollate	None	20	23
(c)	Sodium thioglycollate	None	21	40
	β-mercaptoethanolamine	None	21	49

The reaction mixture consisted of 0.2 ml of fibrinogen solution (2 mg/ml), 0.3 ml buffer saline (120 mg NaCl/100 ml of buffer), 0.1 ml of the aqueous solution of the inhibitor or of equimolar sodium chloride in experiments without inhibitor. The mixture was allowed to stand for 30 sec, at room temperature (28° C) after which 0.4 ml of thrombin was added and the time required to reach the gel point noted.

Concentration of inhibitors in the reaction mixture 10 mM, except in the case of EDTA which was 0.1 mM.

Expt. 1. Canine fibrinogen and calcium free, dilute thrombin; buffer 0.05 M Tris pH 7.4. When calcium or magnesium ions were to be added, 0.05 M Tris buffer pH 7.4 containing 3.4 mM $CaCl_2$ or 1.7 mM $MgCl_2$ was used in place of Tris buffer so that the final concentrations of Ca^{++} and Mg^{++} in the reaction mixture would be 1 mM and 0.5 mM respectively.

2. Human fibrinogen and calcium free dilute thrombin; buffer 0.05 M phosphate pH 7.4.

3. Fibrinogen and buffer as in 1.

(a) Thrombin as prepared by Eagle's method (containing 5 mM Ca^{++}, no further dilution).

(b) Same as above but Ca^{++} removed with an equivalent amount of sodium oxalate.

(c) Same as above but thrombin was diluted ten times with 0.9% sodium chloride.

2. *Effect of Inhibitors on TAME Esterolytic Activity of Thrombin*

Inhibitors with marked effect on the fibrinogen-thrombin reaction viz. glutathione, cysteine, thioglycollate, arginine, and sulphite have been tried for their possible effects on the TAME esterolytic activity of thrombin. They were found to have no inhibitory effect in this regard. Similar results have been obtained in studies with EDTA (Bithel 1964).

3. *Effect of Preincubation of Fibrinogen with Inhibitors on the Enzymatic Phase (Step I)*

It can be seen from Table 2 that preincubation of fibrinogen with EDTA, sulphite and thioglycollate results in a somewhat lower yield of fibrin. Since the possibility of

these inhibitors affecting any other step in the clotting has been corrected for in the control, it would appear that sulphite, EDTA and thioglycollate bring about an alteration of the fibrinogen molecule rendering it less reactive in the clotting process. The effect of these inhibitors on the polymerisation process is however much more marked (vide infra). Arginine or β-mercaptoethanolamine are without any appreciable effect.

Table 2. Effect of Preincubation of Fibrinogen with Inhibitors on Step I of the Fibrinogen-Fibrin Conversion.

Inhibitor	Concentration	% of fibrinogen clotted	
		Test	Control
Arginine	10 mM	51	53
Sodium thioglycollate	10 mM	46	70
Sodium sulphite	10 mM	54	78
EDTA	0.1 mM	43	67
β-mercaptoethanolamine	10 mM	46	50

Strength of thrombin adjusted to give a thrombin time of 20 min. In experiments marked 'Test' 0.2 ml of fibrinogen solution (50 mg/100 ml) was preincubated with 0.3 ml Tris buffer saline (Expt. 1, Table 1) and 0.1 ml of inhibitor solution (1 mM EDTA; 100 mM in case of other inhibitors) for 30 min at the end of which thrombin was added; the action of thrombin was stopped exactly after 10 min by the addition of 0.1 ml of TAME (150 mg/ml water). In 'control' experiments 0.1 ml of sodium chloride of the same molarity as the inhibitor was used in place of inhibitor during preincubation. Following the addition of TAME, 0.1 ml of inhibitor solution was added to 'control' and 0.1 ml of equimolar sodium chloride to 'test'; the reaction mixture was allowed to stand for 30 min to allow polymerisation of fibrin monomer, clot collected by centrifugation and protein estimated.

4. *Polymerisation of Fibrin Monomer*

All the inhibitors of fibrinogen-thrombin reaction are found to inhibit the polymerisation of fibrin monomer (Table 3). The extent of inhibition increases with increasing concentration of inhibitor (Fig. 5).

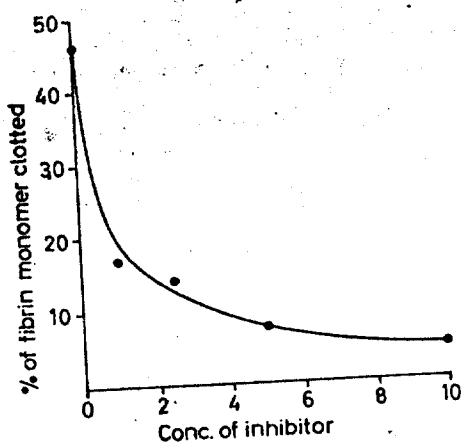

Fig. 5. Effect of concentration of inhibitor on the polymerisation of fibrin monomer. Experimental conditions as in Table 3. Concentration of fibrin monomer 7.5 mg/100 ml, final concentration of inhibitor (glutathione) as indicated (mM). Effect of other thiols, EDTA, sulphite and arginine similar.

Table 3. Effect of Inhibitors on the Polymerisation of Fibrin Monomer.

Expt.	Inhibitor	Additions	% of fibrin monomer clotted
1.	None	None	50
	Cysteine	None	10
	Cysteine	Ca^{++}	43
	β-mercaptoethanolamine	None	9
	β-mercaptoethanolamine	Ca^{++}	43
	Sodium sulphite	None	7
	Sodium sulphite	Ca^{++}	33
	EDTA	None	10
2.	None	None	43
	GSH	None	10
	GSH	Mg^{++}	12
	Sodium thioglycollate	None	10
	Sodium thioglycollate	Mg^{++}	11
3.	None	None	47
	Sodium ascorbate	None	18
	Sodium ascorbate	Ca^{++}	43
	Guanidine	None	28
	Guanidine	Ca^{++}	47
	Arginine	None	13
	Arginine	Ca^{++}	38

The reaction mixture consisted of 4 ml of clotting buffer (phosphate buffer 0.05 M, pH 6.8), 0.5 ml of fibrin monomer solution (75 mg/100 ml) and 0.5 ml aqueous solution of inhibitor (1 mM EDTA or 100 mM in the case of other inhibitors), the reaction was stopped at exactly 5 min by the addition of 1 ml of neutralised 12% w/v formaldehyde. In 'control' experiments without inhibitor, 0.5 ml of an equimolar solution of sodium chloride was used instead. Following the addition of formaldehyde, 0.5 ml of inhibitor solution was added to 'control' tubes and 0.5 ml of equimolar sodium chloride to 'test' tubes. The tubes were allowed to stand for another 30 min, clot collected by centrifugation, washed and protein estimated.

Ca^{++} or Mg^{++} incorporated in the inhibitor, or equimolar sodium chloride used in 'control' experiments as $CaCl_2$ and $MgCl_2$ (10 mM and 5 mM) so as to give a final concentration of 1 mM and 0.5 mM respectively in the reaction mixture.

Fibrin monomer clotted expressed as per centage of its amount used in each experiment.

Calcium ions when present along with the inhibitor largely counteract the inhibition of polymerisation (Table 3). Magnesium ions have only a feeble effect.

5. *Opacity Changes during Polymerisation*

Measurements of opacity of fibrin monomer solution during polymerisation in the presence or absence of inhibitors have been made and the results are shown in Fig. 6. At appropriate concentration of inhibitors the rate of opacification is markedly retarded by thiols, EDTA, arginine and sodium sulphite.

Discussion

Shulman (1951) has pointed out from a comparative study of the effect of -SH compounds and their -OH analogues that the former are likely to be strong inhibitors of the fibrinogen-thrombin reaction. It has been found in this study that besides

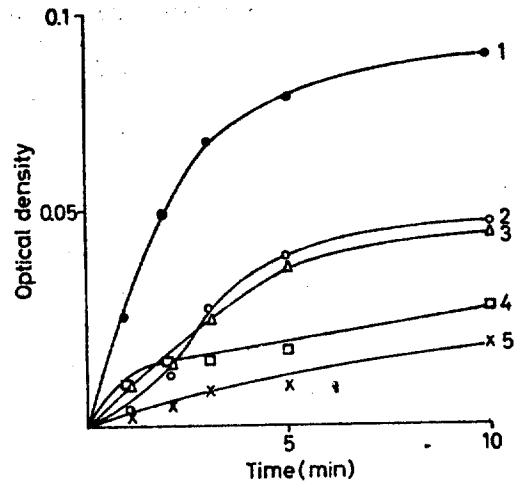

Fig. 6. Optical density of polymerising fibrin monomer solutions in presence of inhibitors. Measurements made in 10 ml cylindrical cuvettes of EEL photoelectric colorimeter at 420 mμ. Reaction mixture as in Fig. 5, but twice the volumes were used. Fibrin monomer concentration 20 mg/100 ml. Final concentration of inhibitors 10 mM except in the case of EDTA which was 0.1 mM. Particular care was taken not to shake the contents of the cuvette to avoid separation of fibrin shreds. 1 = Control; 2 = Arginine; 3 = Sodium thioglycollate; 4 = EDTA; 5 = Sodium sulphite.

cysteine studied by the above author, BAL, GSH, β-mercaptoethanolamine and thioglycollate are strongly inhibitory; thus pointing to a general effect of the -SH group.

It has however been reproved by Fantl and Nance (1947) that thiols do not appreciably inhibit the fibrinogen-thrombin reaction. The possible reasons for this discrepancy may be sought in the conditions under which the inhibitory effect of thiols can be demonstrated. It can be seen from Fig. 1 that the effect of thiols become more and more pronounced when the thrombin used is progressively diluted. The presence of calcium in the thrombin preparation markedly reduces the inhibitory effect of thiols (Fig. 3). Hence if one uses a fairly strong thrombin preparation containing calcium, as it would, if prepared by Eagle's method ,one may nearly miss the effect of thiols at moderately low concentration. The experiments of Fantl and Nance (1947) however differed in two respects from the present work viz. that these authors used human instead of canine fibrinogen and thrombin, and phosphate buffer in place of Tris. Using human material and phosphate buffer the inhibitory effect of thiols could still be demonstrated provided calcium free thrombin at an adequate dilution were used (Table 1).

An inhibitor of the fibrinogen-thrombin reaction may act in 1 of 3 possible ways: (i) by inhibiting thrombin (ii) by altering fibrinogen making it less reactive (iii) by inhibiting the polymerisation of fibrin monomer to fibrin. Shulman (1951) and Bithel (1964) have discounted the possibility of the first mechanism in their studies. Results obtained in this investigation also support this view. Inhibitors like thiols, arginine, and sulphite do not diminish the activity of thrombin towards TAME or fibrinogen. These results are similar to those obtained with EDTA by Bithel (1964). However 3 inhibitors viz. sulphite, thioglycollate and EDTA though without effect on thrombin appear to alter the fibrinogen molecule making it somewhat less reactive with throm-

bin (Table 2). This effect is small compared to the inhibition of the polymerisation phase.

From the foregoing it woulds be seen that the most important mechanism of action of inhibitors is the interference with the polymerisation of fibrin monomer. Direct proof for this is given in Table 3. Inhibitors of polymerisation are also seen to reduce the rate of opacification of polymerising fibrin monomer solutions (Fig. 6).

It has been suggested by previous workers (Ferry and Shulman 1949; Edsall and Lever 1951; Bithel 1964) that inhibitors possibly associate themselves with fibrinogen thereby blocking essential groups involved in polymerisation by their steric interference. The types of possible interactions with fibrinogen or fibrin monomer must be different but specific for each type of inhibitor. But despite the diversity in their chemical nature, the effect of the inhibitors is largely reversed by calcium ions.

The effect of calcium seems to be exercised during the polymerisation phase since the thrombin time of fibrinogen remains the same whether calcium is added along with thrombin or is present during preincubation of fibrinogen with inhibitors. According to Ferry (1954) the effect of inhibitors like hexamethylene glycol is to retard the polymerisation of fibrin monomer especially at Step III. Instead of assuming that the calcium ions neutralise the effect of every type of inhibitor (in the case of EDTA such a neutralisation is possible) a unified explanation may be sought. Calcium ions are known to stabilise fibrin polymers and retard their dissociation in solution (Katz, Shulman, Tinoco, Billick, Gutfreund and Ferry 1953). Probably calcium ions shift the dissociation equilibrium between fibrin monomer and fibrin clot in favour of the latter and thereby counteract the effect of the inhibitors on polymerisation. Calcium ions are also known to be important in the formation of intermolecular bonds responsible for the insolubility in urea and the physical characteristics of plasma fibrin (Biggs and MacFarlane, 1962; Loewy, Gallant and Dunathan, 1961) but this effect cannot explain the observations made in this study since the clot formed from fibrin monomer in presence of calcium is soluble in 5 M urea.

Calcium ions do not appear to be essential for the fibrinogen thrombin reaction but can hasten the process of clotting. It would therefore appear that in the process of coagulation of blood, ionic calcium is most likely to play a favourable role apart from the activation of thrombokinase.

Summary

1. The inhibitory effect of a number of substances on the fibrinogen-thrombin reaction has been studied. The effect is best observed at pH 7.4 using calcium free dilute thrombin. Ca^{++} reduces the effect; Mg^{++} is less effective than Ca^{++}. The optimum concentration of Ca^{++} is 0.5 to 1 mM.
2. Preincubation with fibrinogen increases the effect of EDTA, sulphite and thioglycollate on the thrombin time. These compounds also appear to alter fibrinogen and thereby retard the conversion of fibrinogen to fibrin monomer.
3. Preincubation of thrombin with these substances does not result in loss of TAME esterolytic activity or in prolongation of thrombin time of fibrinogen.
4. Inhibitors of the fibrinogen-thrombin reaction retard the polymerisation of fibrin monomer to fibrin and also the attendant increase in opacity.
5. The inhibitors of the fibrinogen-thrombin reaction studied appear to exert their effect mostly at the stage of polymerisation of the fibrin monomer. Calcium ions largely reverse the inhibition of this step.

Résumé

1. On a étudié l'effet inhibiteur d'un certain nombre de substances sur la réaction fibrinogène – thrombine. L'effet est observé de façon optimale au pH 7,4 avec une thrombine diluée sans calcium. Ca^{++} diminue l'effet inhibiteur; Mg^{++} est moins actif que Ca^{++}. La concentration optimale en Ca^{++} se situe entre 0,5 et 1 mM.

2. La préincubation avec le fibrinogène augmente l'effet de l'EDTA, du sulfite et du thyoglycollate sur le temps de thrombine. Ces substances semblent altérer le fibrinogène et par voie de conséquence retardent la conversion du fibrinogène en monomère de fibrine.

3. La préincubation de ces mêmes substances avec la thrombine ne provoque pas de perte d'activité estérolytique, testée sur TAMe, ni une prolongation du temps de thrombine mesuré avec le fibrinogène.

4. Les inhibiteurs de la réaction fibrinogène – thrombine retardent la polymérisation du monomère en fibrine et l'augmentation concomittante de l'opacité du caillot.

5. Les inhibiteurs de la réaction fibrinogène – thrombine semblent exercer leur action principale au moment de la polymérisation du monomère de fibrine. Les ions calciques renversent grandement cette inhibition.

Zusammenfassung

1. Es wurde die Hemmwirkung einer Reihe von Substanzen auf die Fibrinogen-Thrombin-Reaktion untersucht. Die beste Wirkung wird bei pH 7,4 unter Verwendung von Ca-freiem, gelöstem Thrombin beobachtet. Ca^{++} schränkt die Wirkung ein; Mg^{++} ist weniger wirksam als Ca^{++}. Die optimale Konzentration von Ca^{++} liegt zwischen 0,5 und 1 mM.
2. Die Vorinkubation mit Fibrinogen verstärkt die Wirkung von EDTA, Sulfit und Thioglykollat auf die Thrombinzeit. Diese Substanzen scheinen auch Fibrinogen zu verändern und dadurch die Verwandlung von Fibrinogen zu Fibrinmonomer zu verzögern.
3. Die Vorinkubation von Thrombin mit diesen Substanzen resultiert nicht in einem Verlust von esterolytischer TAME-Aktivität oder in einer Verlängerung der Thrombinzeit des Fibrinogens.
4. Hemmer der Fibrinogen-Thrombin-Reaktion verzögern die Polymerisation des Fibrinmonomers zu Fibrin und die gleichzeitig entstehende Zunahme der Trübung.
5. Die Hemmer der untersuchten Fibrinogen-Thrombin-Reaktion scheinen ihre Wirkung am besten im Stadium der Polymerisation des Fibrinmonomers zu entfalten. Kalziumionen machen die Hemmung dieser Stufe weitgehend rückgängig.

Acknowledgement

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(German Publication, pp. 707-8)

Inhibition of esterase systems by fat antioxidants

Antioxidants which inhibit the reaction chain of lipo-peroxidation are used in food technology to stabilize fats. These additives get into the body with the food and, after resorption, into the bloodsteam and into various organs. Being lipo-soluble, the antioxidants accumulate mainly in the tissues whose cells contain relatively high proportions of fat (e.g. in the fatty tissue, in the liver) (1).

The antioxidants used in the practice belong mainly to the phenol type. On interruption of the reaction chain of autoxidation, the phenol group is oxidized to quinone; this process is reversible. This makes it possible for the anti-oxidants to influence the biologic oxidation-reduction processes, e.g. the dehydrogenase and oxidase systems (2). We investigated this influence in the esterase systems of the serum, of the liver, and of the fatty tissue; a fundamental effect of their redox potential cannot be presumed with these observations.

For the tests we used the serum of fasting rats; the male Wistar rats were left without food for 16 hours. The liver homogenate was prepared by formulating 1 part liver tissue with 9 parts veronal-acetic acid buffer, pH 7.9, or respectively by taking from the perirenal or epididymis fatty tissue homogenate 1 part fatty tissue with 3 parts of the buffer. The substrate consisted of polyoxyethylene sorbito-mono-oleate (Tween 80) in the veronal-acetic acid buffer, pH 7.9, in the presence of calcium. The antioxidants were first dissolved in Tween; the values listed in Table 1 correspond to the antioxidant concentration in the incubate; incubation time 60 min for serum (0.1 ml) or liver homogenate (0.1 ml), or 120 min for fatty tissue homogenate (0.2 ml). The nephelometric measurement is based on observation of the quantity of cal-

cium oleate formed (3).

For control, blind tests were carried out so as to eliminate the effect of the antioxidants on the formation of turbidities; at the same time, with partial condensation of the octyl gallate, the required correction was made (a faintly green-brown discoloration of the ellagic acid). Since with regard to the substrate preparation it may be assumed that the lipo-soluble antioxidants inhibit the esterolysis only physically (by adhesion to the lipid parts of the Tween molecules), we made comparisons with similarly prepared substrate containing compounds of an similar nature, e.g. with cholesterol; the result was not influenced with respect to the possibility stated. The findings are indicated as activity of the enzyme systems (millimole of oleate formed per min and per g of tissue or per ml of serum) as well as relatively as inhibition intensity in % (Table 1).

Table 1. Inhibition of the esterase systems by fat antioxidants.

	Conc. (millimole)	Esterase system							
		Serum		Liver		Perirenal fatty tissue		Epididymis fatty tissue	
		a	b	a	b	a	b	a	b
Noridhydroguayaretic acid	0.0	3.43	0.0	26.86	0.0	0.95	0.0	1.75	0.0
	0.1	2.68	21.9	26.69	0.6	1.02	-7.0	1.72	1.9
	0.5	2.07	39.8	26.01	3.0	0.92	3.5	1.73	1.0
	1.0	0.64	81.3	3.4	87.4	0.98	-3.5	1.73	1.0
Butyl hydroxytoluene	0.0	3.35	0.0	27.03	0.0	1.00	0.0	1.03	0.0
	0.1	3.10	7.6	23.46	13.1	1.00	0.0	1.05	-1.6
	0.5	3.10	13.2	22.78	15.7	0.98	1.7	1.03	0.0
	1.0	1.32	60.7	7.14	73.5	0.97	3.3	1.02	1.7
Ascorbyl palmitate	0.0	3.35	0.0	28.05	0.0	1.42	0.0	1.27	0.0
	0.1	2.72	18.9	25.67	8.5	1.43	-1.2	1.27	0.0
	0.5	2.32	31.0	25.33	9.1	1.48	-4.7	1.20	5.3
	1.0	0.32	80.5	9.52	66.1	1.42	0.0	1.20	5.3
Octyl gallate	0.0	3.32	0.0	26.35	0.0				
	0.1	2.68	10.1	24.82	5.8				
	0.5	2.72	18.1	23.12	12.1				
	1.0	0.28	91.46	20.06	23.8				

a - Activity (millimole/g/min)

b - Inhibition (%)

The antioxidants affect the various esterase systems differently. The serum esterases are strongly inhibited in all cases. The decrease in activity of the liver esterases is much less with octyl gallal than with the other antioxidants. The esterolytic activity of the fatty tissues is relatively low and is not significantly influenced by antioxidants. It is clearly evident from the findings that the tested antioxidants inhibit the esterolytic serum and liver systems and thus also can influence the lipid metabolism. More exact analyses of the respective esterase types will be carried out later.

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Hemmung von Esterasen-Systemen durch Fett-Antioxydantien

Ascorbyl Palmitate #43

Antioxydantien, die die Reaktionskette der Lipoperoxydation hemmen, werden in der Lebensmitteltechnologie zur Stabilisierung der Fette verwendet. Mit der Nahrung gelangen diese Zusatzstoffe in den Organismus und nach der Resorption in die Blutbahn sowie in verschiedene Organe. Da die Antioxydantien fettlöslich sind, so häufen sie sich hauptsächlich in den Geweben an, deren Zellen höhere Fettanteile enthalten (z. B. im Fettgewebe, in der Leber) [1].

Die in der Praxis verwendeten Antioxydantien gehören hauptsächlich dem Phenoltypus an. Bei Unterbrechung der Reaktionskette der Autoxydation ist die Phenolgruppe zum Chinon oxydiert; dieser Prozeß ist reversibel. Damit ist die Möglichkeit gegeben, daß die Antioxydantien die biologischen Oxydations-Reduktions-Vorgänge beeinflussen, z. B. die Dehydrogenasen- und Oxydasen-Systeme [2]. Wir verfolgten diesen Einfluß bei den Esterasen-Systemen des Serums, der Leber und des Fettgewebes; einen grundsätzlichen Effekt ihres Redox-Potentials kann man bei diesen Beobachtungen nicht vermuten.

Zu den Versuchen verwendeten wir das Serum nüchterner Ratten; die männlichen Wistar-Ratten hungerten 16 Std. Das Leberhomogenat wurde vorbereitet, indem 1 Teil Lebergewebe mit 9 Teilen Veronal-Essigsäure-Puffer, pH 7,9, bzw. vom Perirenal- oder Epididymis-Fettgewebe-Homogenat 1 Teil Fettgewebe mit 3 Teilen des Puffers angesetzt wurden. Das Substrat bestand aus Polyoxyäthylensorbitmonooleat (Tween 80) im Veronal-Essigsäure-Puffer, pH 7,9, in Gegenwart von Calcium. Die Antioxydantien wurden zuerst in Tween gelöst; die in der Tab. 1 angeführten Werte entsprechen der Antioxydantienkonzentration im Inkubat; Inkubationsdauer: 60 Min. bei Serum (0,1 ml) bzw. Leberhomogenat (0,1 ml) oder 120 Min. bei Fettgewebehomogenat (0,2 ml). Die nephelometrische Messung gründet sich auf die Erfassung der entstandenen Calciumoleat-Menge [3].

Tabelle 1
Hemmung der Esterasen-Systeme durch Fett-Antioxydantien

Antioxydant	Konzentration [mMol]	Esterasen-System: Serum		Leber		Perirenal-Fettgewebe		Epididymis-Fettgewebe	
		Aktivität [mMol/g/min]	Inhibition [%]	Aktivität [mMol/g/min]	Inhibition [%]	Aktivität [mMol/g/min]	Inhibition [%]	Aktivität [mMol/g/min]	Inhibition [%]
Nordihydroguajaretsäure	0,0	3,43	0,0	26,86	0,0	0,95	0,0	1,75	0,0
	0,1	2,68	21,9	26,69	0,6	1,02	−7,0	1,72	1,9
	0,5	2,07	39,8	26,01	3,0	0,92	3,5	1,73	1,0
	1,0	0,64	81,3	3,4	87,4	0,98	−3,5	1,73	1,0
Butylhydroxytoluol	0,0	3,35	0,0	27,03	0,0	1,00	0,0	1,03	0,0
	0,1	3,10	7,6	23,46	13,1	1,00	0,0	1,05	−1,6
	0,5	2,90	13,2	22,78	15,7	0,98	1,7	1,03	0,0
	1,0	1,32	60,7	7,14	73,5	0,97	3,3	1,02	1,6
Ascorbylpalmitat	0,0	3,35	0,0	28,05	0,0	1,42	0,0	1,27	0,0
	0,1	2,72	18,9	25,67	8,5	1,43	−1,2	1,27	0,0
	0,5	2,32	31,0	25,33	9,1	1,48	−4,7	1,20	5,3
	1,0	0,32	80,5	9,52	66,1	1,42	0,0	1,20	5,3
Octylgallat	0,0	3,32	0,0	26,35	0,0				
	0,1	2,68	19,1	24,82	5,8				
	0,5	2,72	18,1	23,12	12,1				
	1,0	0,28	91,46	20,06	23,8				

Zur Kontrolle wurden Blindversuche durchgeführt, um den Einfluß der Antioxydantien auf die Bildung der Trübungen ausschalten zu können; zugleich wurde bei teilweiser Kondensation des Octylgallates die erforderliche Korrektur durchgeführt (eine schwach grünbraune Verfärbung der Ellagsäure). Da bezüglich der Substratvorbereitung zu vermuten ist, daß die fettlöslichen Antioxydantien die Esterolyse nur physikalisch hemmen (durch Adhäsion an den Lipidteilen der Tween-Moleküle), führten wir mit in gleicher Weise vorbereitetem Substrat, das Verbindungen ähnlichen Charakters enthält, Vergleiche durch, z. B. mit Cholesterin; das Ergebnis war in bezug auf die angegebene Möglichkeit nicht beeinflußt. Die Befunde werden als Aktivität der Enzymsysteme (mMol entstandenes Oleat je 1 Min. und je 1 g Gewebe bzw. je 1 ml Serum) sowie relativ als Inhibitionsintensität in % angegeben (Tab. 1).

Die Antioxydantien wirken auf die einzelnen Esterasen-Systeme verschieden. Die Serumesterasen werden in allen Fällen stark gehemmt. Die Aktivitätsabnahme der Leberesterasen ist bei Octylgallat gegenüber den anderen Antioxydantien wesentlich geringer. Die Fettgewebe weisen nur eine relativ niedrige esterspaltende Aktivität auf, die durch Antioxydantien statistisch nicht signifikant beeinflußt wird. Aus den Befunden geht eindeutig hervor, daß die geprüften Antioxydantien die esterspaltenden Serum- und Lebersysteme hemmen und dadurch auch den Fettstoffwechsel beeinflussen können. Genauere Analysen der jeweiligen Esterasentypen werden erst später durchgeführt.

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THIAMINE-SPARING ACTION OF ASCORBIC ACID ON SOME *LACTOBACILLI* AND THE RAT DUE TO THIAMINE DISULFIDE ACTIVATION*

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Stimulatory interaction between ascorbic acid and other water-soluble vitamins belonging to the B group has been repeatedly observed during the last two decades in conjunction with nutritional studies of both animal and microbial species. The explanation offered for such phenomena was usually twofold, as pointed out by Reid (1954) in her excellent review. Thus it was concluded generally that ascorbic acid augments the biosynthesis of some B vitamins. On the other hand, at least thiamine, pantothenic acid, and riboflavin were believed to stimulate independently the synthesis of ascorbic acid in some tissues. Consequently both views were accepted for thiamine, a factor in which I became interested recently chiefly because of the inconsistent data available on the utilization of its various forms.

Observations on the synergistic action of thiamine and ascorbic acid were made as far back as 1939 when Kasahara and his co-workers injected ascorbic acid and thiamine subcutaneously into young rats and obtained a growth enhancement. With the same levels of thiamine administered alone (0.2 μg. daily thiamine per animal), the animals lost weight. On the other hand, the growth with both factors administered simultaneously matched the rich growth obtained with five-times-higher thiamine concentrations in the absence of ascorbic acid. Pigeons and guinea pigs behaved as did the rat. Independently of the Japanese authors, the Italian authors Aloisi and Polanyi (1939, 1940) found that the neurological syndromes of thiamine-deficient rats ceased immediately after administration of ascorbic acid.

Further confirmation of these findings came when Daft and Schwarz (1952) reported the prevention or delay of onset of deficiency signs in rats on thiamine-free diets when 5 per cent ascorbic acid was added to the diet. This observation was made in connection with studies on the prevention of pantothenate-deficiency syndromes with 2 per cent ascorbic acid.

These observations might have been considered casual and thus might have received less attention than the interaction of ascorbic acid with the other vitamins if microbiological reports had not claimed similar growth stimulation at low thiamine levels. Thus Fang and Butts (1951, 1953) described in detail the thiamine-sparing action of ascorbic acid for the growth of the heterofermentative organism *Lactobacillus fermenti* strain 36.

The authors discussed the possibilities of ascorbic acid action and rejected the idea of a simple reducing effect. Instead it was concluded that ascorbic acid participates in pyruvate metabolism as a cofactor by allowing *L. fermenti* cells to bypass the particular steps of glucose metabolism that are inhibited by

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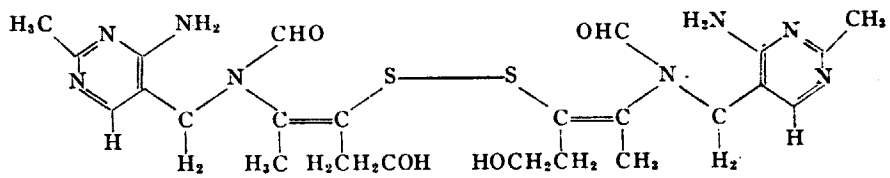
fluoride. Although not referred to by Cavallini and his co-workers (1946), this suggestion accorded with their ideas on the direct involvement of ascorbic acid in pyruvate decarboxylation. Based on thiochrome assay of thiamine content of *L. fermenti* in the presence of either thiamine or ascorbic acid, or of both compounds, Fang and Butts (1952) also concluded that ascorbic acid is directly involved in the synthesis of thiamine by the bacterial cells.

A straightforward attempt to coordinate the microbiological and rat nutritional work on thiamine-sparing action of ascorbic acid was made recently by Terroine (1957) as a pursuit of her basic studies on nutritional vitamin interrelations (Terroine, 1955). In reference to the present discussion the accurate establishment of some peculiarities of this interrelationship are of basic importance. Thus it was shown that D-*iso*-ascorbic acid exerted the same protective action on both *L. fermenti* and the rat as did L-ascorbic acid, despite the lack of vitamin-C activity of the former. We should also consider Terroine's finding that the protective effect of ascorbic acid in thiamine deficiency in the rat was only of a temporary nature and that it led to a regression after about 6 to 8 weeks; moreover ascorbic acid was unable to protect the hepatic or other tissue thiamine reserves from exhaustion.

This was the state of affairs when I started work on the nutritional requirements of *L. fermenti*. My aim was by no means directed toward ascorbic acid; I was trying merely to find a more accurate microbiological estimation of thiamine. After changing the basal medium for thiamine assay with *L. fermenti* in both fluid (Bánhidi, 1958) and agar-plate test (Bánhidi, 1959), I found that the quantitative thiamine values still varied in some experiments in an unexplained and discouraging way. Much time passed before the main source of error could be identified as variations in the proportions of reduced and oxidized state of thiamine (Bánhidi, 1959*a*).

The reason these variations affected the growth response was traced to the nutritional unavailability of the oxidized form of thiamine disulfide:



Specific experimental conditions were required to demonstrate the full extent of inactivity of thiamine disulfide when compared with thiamine. In the basal medium, which I formulated, the alkaline-treated peptone supplement was replaced by increased amounts of acid-hydrolyzed casein and tryptophan. Additional cystine and cysteine were not added to the medium since *L. fermenti* does not need these factors for growth; nevertheless the medium contained these amino acids in small amounts that were not sufficient to interfere with the experiments. The fact that Koser and Thomas (1955) firmly established the dispensability of cystine and cysteine also for this strain of *L. fermenti* was overlooked in most of the work on thiamine assay with this organism, including work described in our own earlier papers. This is another

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example of how specialization in science can sometimes render experimentation as well as interpretation of the results more complex.

After introduction of these technical changes and through aseptical addition of the thiamine and thiamine disulfide solutions to the autoclaved medium, I obtained (Bánhidi, 1959*a*, 1960) a clear-cut answer on the inactivity of thiamine disulfide (FIGURE 1a). Activation of this compound was easily achieved

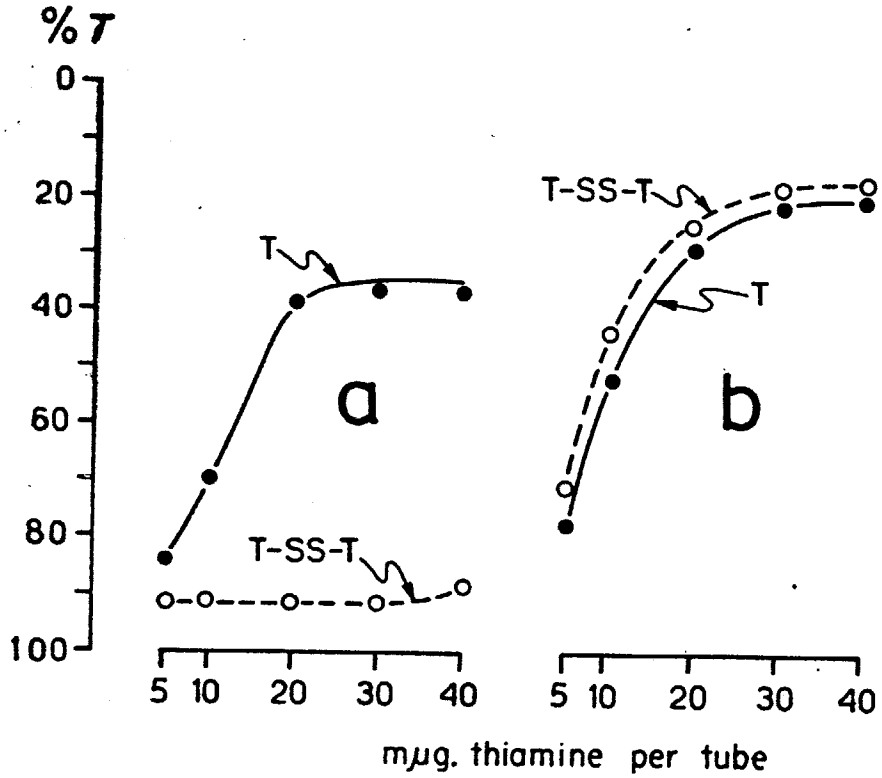

FIGURE 1. Growth of *Lactobacillus fermenti* on a chemically defined medium with thiamine (T) and thiamine disulfide (T—SS—T): (*a*) in the absence of cysteine and (*b*) with cysteine added to the medium (500 μg./ml. final volume). Turbidity readings represent mean values of duplicates or triplicates and are expressed as percentage of light transmission (% *T*) at 5950 A (corresponding optical density = *D* = −log *T*). Reproduced by permission of the *Journal of Bacteriology*.

through the addition of suitable amounts of cysteine to the cultures (FIGURE 1b). In this way also the disulfide present in the thiamine standard solutions became activated, resulting in a typical "stimulatory" effect (cf. the curves of T in FIGURES 1a and 1b).

We now know that the effect of cysteine activation of thiamine disulfide is not necessarily a simple reduction of the oxidized form to the thiazole form, since fully active thiamine-cysteine mixed disulfide might also be formed under suitable conditions (Bánhidi, 1960*c*). A reduction of the disulfide form by boiling, autoclaving, or through chemical agents, however, resulted in the

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same activation. Thus L-ascorbic acid (FIGURES 2a and 2b) and D-*iso*-ascorbic acid were shown to liberate thiamine activity from thiamine disulfide, and the activity was evidently dependent on the concentrations of the ascorbic acids (FIGURES 2a and 2b) since it was the function of concentration in the case of cysteine. Naturally, similar activation could be obtained also with lower concentrations of triose reductone (von Euler, 1957), as I had reported (Bánhidi, 1960).

The next step was a nutritional trial on the rat *per analogiam* to the work of Terroine (1957) since I felt that all her results should be regarded as strong indications that ascorbic acid mobilized some scarce nutritional or endogenous inactive forms of thiamine rather than replaced thiamine by ascorbic acid in intermediary processes. In our first experiments either both forms of thiamine or only thiamine disulfide plus ascorbic acid promoted growth, depending on whether too-high or too-low molar concentrations of the thiamine derivatives were used. Finally a curative dose of 500 μg./kg. diet was found, which allowed us to demonstrate the necessity of activating the disulfide form of thiamine for the rat (FIGURE 3). This was shown with Wistar rats kept on a semisynthetic diet containing 68 per cent sucrose and 20 per cent vitamin-free casein after a depletion period of 2 weeks and by protection of the animal from coprophagy (Bánhidi, 1960*b*).

After the suitable concentration range for thiamine was established the experimental growth curves were easily reproduced, and I obtained a system in which the nutritional value of other thiamine derivatives, particularly those of mixed disulfides as well as those of some O- and S-substituted analogues, could be tested. By definition this should be done "under conditions in which thiamine disulfide requires activation for permanently curing the thiamine deficiency syndromes of the rat." FIGURE 4 (unpublished data) shows that under certain conditions in allithiamine derivative, thiamine propyl disulfide was found to be active without requiring the addition of ascorbic acid (cf., Bánhidi, 1960*d*).

This result was of particular interest because of the pharmacological use of the thiamine propyl disulfide and because of the inactivity of this compound toward different *Lactobacilli* requiring thiamine (Bánhidi, 1959*b*; 1959*c*; 1960*a*).

When raising the question of why the redox action of ascorbic acid on thiamine derivatives was more or less rejected by both Fang and Butts (1953) and Terroine (1957), one cannot escape the feeling that the reductones propagated by von Euler (1951) might deserve much wider attention in nutritional trials (Bánhidi and von Euler, 1951). This is particularly recommended in a study with ascorbic acid (Bánhidi, 1960). This retrospective question does not imply a criticism of earlier work on such intricate problems. On the contrary, it should be pointed out that the inactivity of the disulfide form of thiamine in bacterial cells and higher organisms was not known at that time. It was also believed that thiamine disulfide was more active as a nutrient than the homomeric form of this vitamin (Zima *et al.*, 1953). The paradoxical fact is that this early statement might be apparently true if the batch of recrystallized thiamine disulfide were purer than the thiamine used for comparison, provided that the disulfide form was activated in that particular test through some reduction (Bánhidi, 1960). Naturally, this statement disregards the fact that

the disulfide should have been tested in its intact form in order to establish its nutritional value. The situation was even more complicated in the microbial test because of the presence of some cysteine and large amounts of cystine added to the basal medium. The latter was easily reduced by heat-sterilizing the medium.

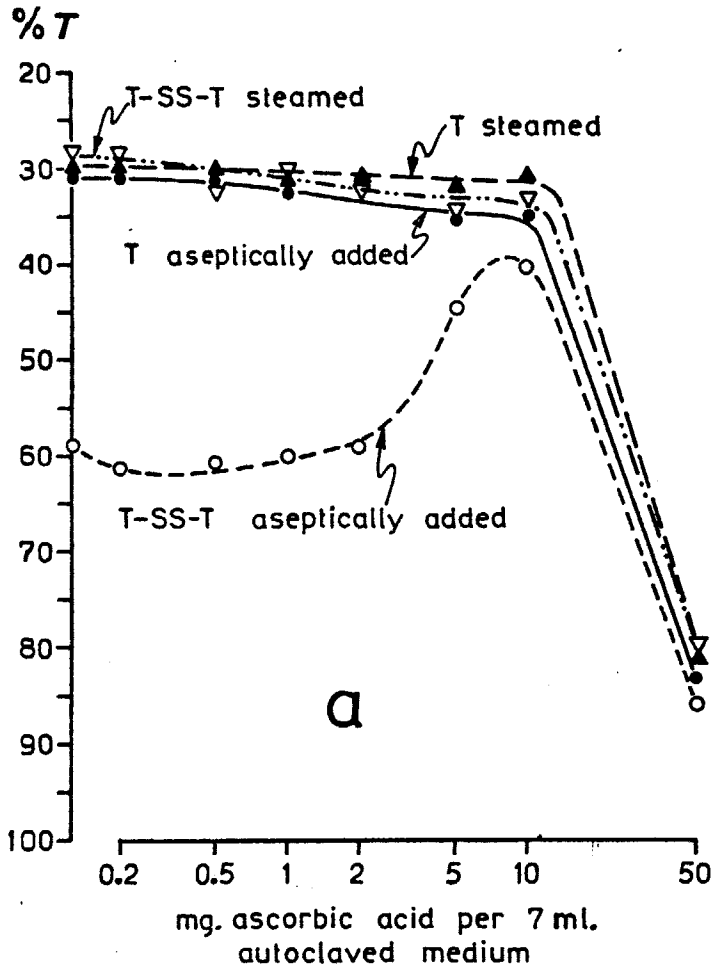

FIGURE 2. (*a*) Activation of thiamine disulfide (T—SS—T) to growth factor toward *Lactobacillus fermenti* by increasing concentrations of ascorbic acid in the medium. Thiamine (T) and reduced thiamine and thiamine disulfide through steaming with the medium served as controls. Reproduced by permission from the *Journal of Bacteriology*. (*b*), p. 329 The concentration dependence of the activation was also demonstrated with *iso*-ascorbic acid (*iso*-Asc.Ac.). Reproduced by permission from *Zeitschrift für Vitaminforschung*. In both *a* and *b* constant levels of 20 mμg. thiamine (T) or 18.8 mμg. thiamine disulfide (T—SS—T) were applied. The drastic fall of the curves after the highest points of activation is due to strong inhibitory action of the ascorbic acid on the bacterial growth at higher concentrations. Turbidity readings represent mean values of duplicates or triplicates and expressed as percentage of light transmission (% *T*) at 5950 Å (corresponding optical density = $D = -\log T$).

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In an early work on the nutrition of the rat, the homomeric disulfide of thiamine was found to be much less active than thiamine (Zima *et al.*, 1953). However, as I pointed out (Bánhidi, 1960*b*) at the time, no specific attention

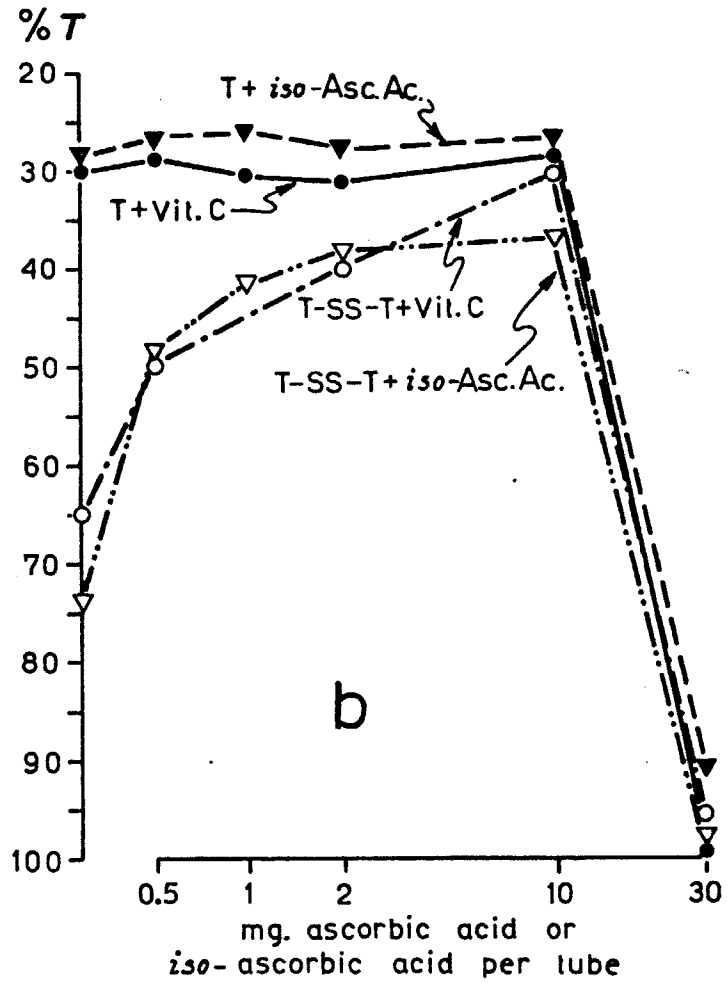

FIGURE 2.

was paid to this important fact, which stood out from a great volume of data that seemingly supported evidence contrary to this single observation.

While I believe that the disulfide activation phenomena explain some of the questions of nutritional thiamine-ascorbic-acid interaction, we must face at least two consequences: on the one hand, the results presented above may point to a useful way to study other thiamine derivatives and other B vitamins with respect to ascorbic acid synergism; on the other hand, the necessity of similar studies performed on germfree animals can hardly be overemphasized.

In one of my discussions (Bánhidi, 1960*c*), I stated that "convection" (nutritional features overshadowed by intestinal symbiosis) should not interfere with the demonstration of the inactivity of homomeric thiamine disulfide in the rat. This is because in my work a negative effect could be shown that pointed to the inability of the rat and its intestinal flora either to utilize or to

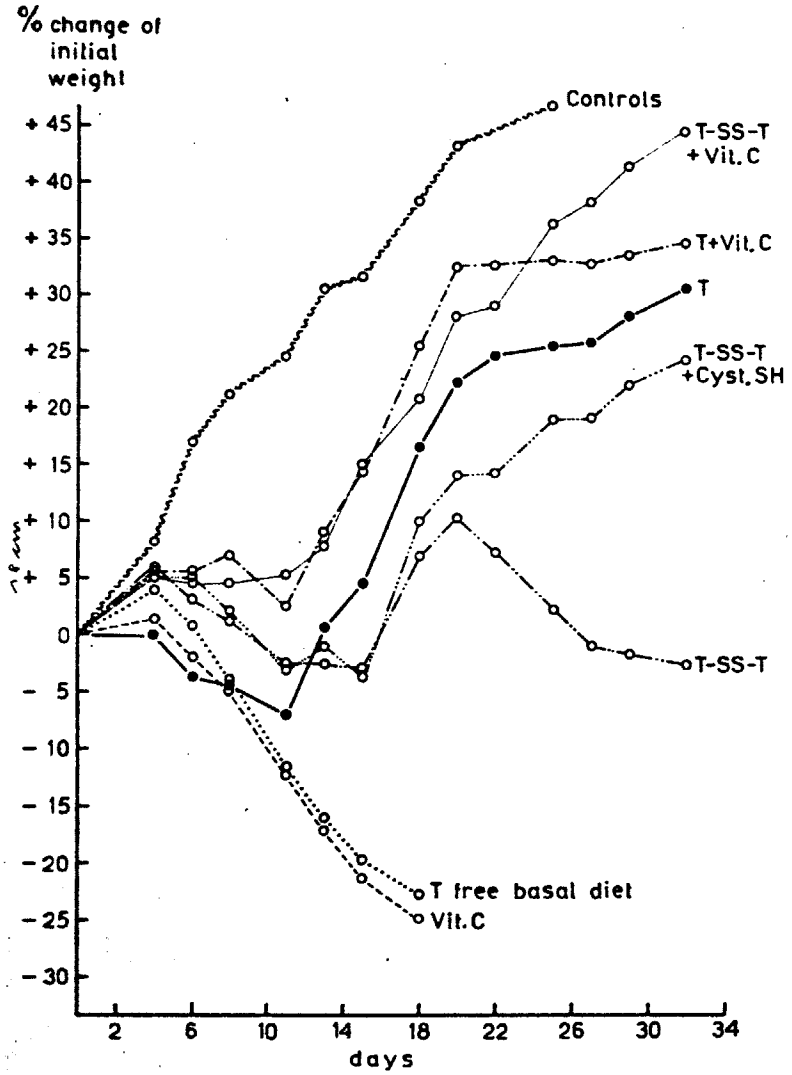

FIGURE 3. Activation of dietary thiamine disulfide (T—SS—T) by ascorbic acid (vit. C) or cysteine (Cyst.SH) for growing male albino rats (Wistar strain) as compared with growth with and without added thiamine (T) on an equimolar basis calculated as thiamine thiole. Weight gains or losses are expressed as percentage changes in total weight after a 2-week total thiamine-depletion period and were plotted against time (Bánhidi, 1960*b*). Reproduced by permission from the *Internationelle Zeitschrift für Vitaminforschung*.

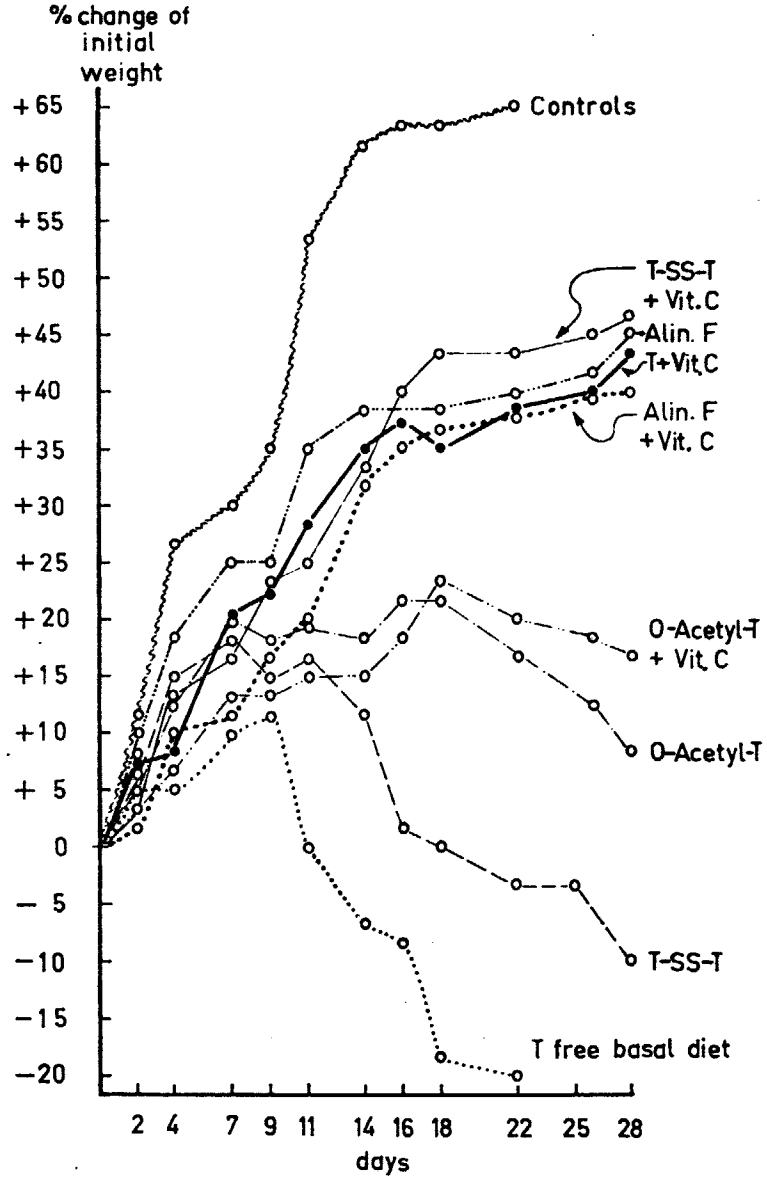

FIGURE 4. Rat growth experiment as in FIGURE 3. A mixed disulfide of the allithiamine type (thiamine propyl disulfide = "Alinamine F" of the Takeda Pharmaceutical Industries, Ltd. [Osaka, Japan] = Alin. F) was found to be active under conditions when thiamine disulfide is inactive for the rat. Purified *o*-acetyl thiamine (*o*-Acetyl-T) shows only limited activity because of the liberation of some thiamine impurities, and simultaneous addition of ascorbic acid could not result in the activation of this analogue.

reduce the disulfide form. This is, however, an exceptionally unique case, and it will be rather difficult to present similar unanimous explanations when studying active derivatives (for example, allithiamine). In these cases we must consider the action of intestinal flora as well as specific resorption conditions through the intestinal mucosa of the animals. This is indeed a field offering many problems to the nutritionist.

I certainly agree with Brown and Sturtevant (1949), who pointed out a decade ago that germfree studies are required to decide such questions as the role of intestinal synthesis of ascorbic acid—if any—for animals able to perform endogenous tissue synthesis. Concerning ascorbic acid and thiamine interaction in germfree animals, the problem of designing experiments must take into account the fact that a potential antimetabolite, oxythiamine, is formed during steam sterilization. According to Wostmann (1959) this renders thiamine one of the most vulnerable components in respect to both steam and radiation sterilization, and it represents a crucial point in germfree animal nutrition experiments.

Acknowledgment

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served in animals pretreated with either metopirone or Su-9055.

These findings therefore support the suggestion of WONG and WARNER[8] that interruption of the synthetic pathway of adrenal corticoids is not the only way by which DMBA-induced necrosis can be prevented and that competition between Su-9055 and DMBA at the tissue sites is an important factor. However, we should like to modify this hypothesis, and propose that competition for specific receptor sites in the adrenals occurs between the metabolites of DMBA, metopirone and Su-9055 rather than the original compounds. Thus, there is now strong evidence[14,15] that the proximal necrotic agent is the 7-hydroxymethyl derivative of DMBA, and it is also known that metopirone is converted to a secondary alcohol by rat adrenals. In this respect, it is of interest that Su-9055 also contains a keto group which might therefore be reduced in rat tissues[17].

Résumé. Nous avons protegé des rats contre l'action destructive du DMBA sur les adrénales avec des doses de Su-4885 ou de Su-9055 qui n'avaient pas d'effet sur le métabolisme du progestérone et du déoxycorticostérone. Un mécanisme expliquant l'action de ces substances inhibitrices est suggéré.

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Cancer Research Centre, University of British Columbia, Vancouver (Canada), 17 October 1967.

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Ascorbic Acid Oxidizes Thiol Groups of Plasma Proteins

Although ascorbic acid usually functions as a reducing agent, it can oxidize thiols such as glutathione[1]. Oxidation of protein thiol groups by ascorbic acid has not been reported. I have observed oxidation of the thiol groups of human plasma proteins by ascorbic acid in vitro at physiological pH.

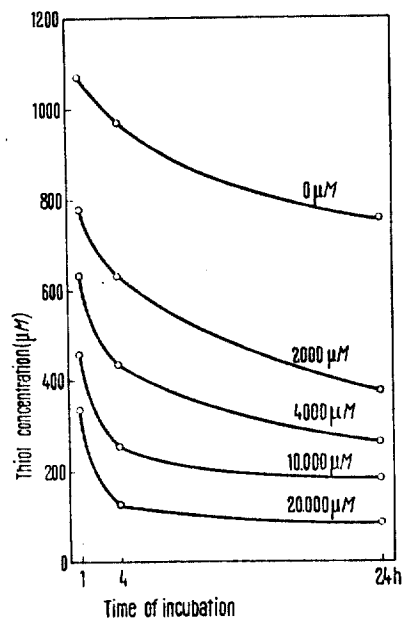
Outdated human blood was obtained from the blood bank, and the plasma was separated by centrifugation. The plasma contained acid citrate dextrose (ACD) anticoagulant.

L-Ascorbic acid was dissolved to make a 0.2 *M* solution in phosphate buffer, and the pH was adjusted to 7.0 with sodium hydroxide solution. An appropriate volume of the ascorbic acid solution was added to 9 ml of plasma, buffer was added to bring the final volume to 10 ml, and the mixture was incubated at 20 °C. Several incubations were done at each concentration of ascorbic acid, with good reproducibility. The same results were obtained when D-araboascorbic acid was used instead of L-ascorbic acid.

Thiol concentrations were measured with 5, 5'-dithiobis(2-nitrobenzoic) acid according to the method of ELLMAN[2]. This reagent reacts with both reactive and 'unreactive' thiol groups.

I observed a rapid decrease in the thiol concentration as soon as the ascorbic acid was added. The thiol concentration continued to decrease more slowly for at least 24 h afterwards, as shown in the Figure. This decrease in the thiol concentration is the result of oxidation of thiol groups to disulfide bonds[3]. The rate of oxidation was greater when the concentration of ascorbic acid was increased.

The rapid initial oxidation probably involved the reactive thiol groups, and the slower oxidation that followed probably involved 'unreactive' thiol groups that became accessible to the ascorbic acid as the protein gradually became denatured[3]. A slow oxidation of thiol groups was observed even in the absence of ascorbic acid, as shown by the top curve in the Figure. Because the concentration of glutathione in the plasma is negligible, the major effect of ascorbic acid must be on the protein fraction of the plasma.



Decrease in total thiol concentration of human blood plasma upon incubation with ascorbic acid. The curve labels show the concentrations of ascorbic acid.

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The oxidative ability of ascorbic acid may be important in vivo; for example, in regulating biologically active proteins such as antibodies and enzymes. Additional experiments will be done in this field.

Zusammenfassung. Ascorbinsäure oxydiert die Thiolgruppen der Proteine im Plasma menschlichen Blutes schnell. Diese Fähigkeit der Ascorbinsäure mag für biologische Systeme grössere Bedeutung haben.

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Distribution of Inulin-carboxyl-C^{14} in Heart and Skeletal Muscle with Respect to in vivo and in vitro Extracellular Space Determinations

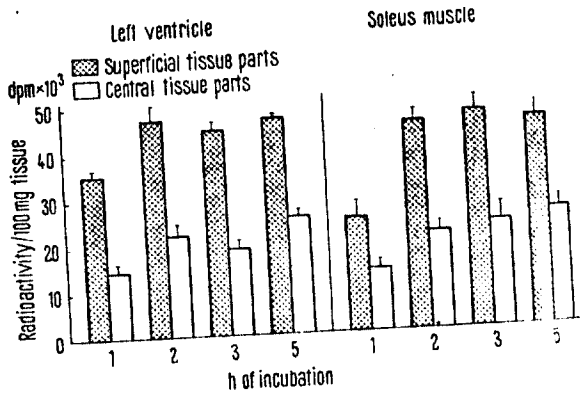
Methods for the determination of extracellular space are based on the same principle, namely the uniform distribution of an indicator throughout the entire extracellular space by the blood stream in vivo and from an incubation fluid in vitro[1]. Inulin is widely used as such an indicator since it is not metabolized and does not penetrate cells as shown by autoradiography[2] and by an osmotic gradient method[3]. In vivo experiments are generally done with nephrectomized animals; equilibrium between the serum and the extracellular space is believed to occur when the serum indicator concentration after i.v. injection falls to a constant level. After the extracellular space values obtained reach a plateau, when plotted against time, it is assumed that an equilibrium in vitro between incubation medium and extracellular space is achieved. This latter equilibration depends on the size and type of tissue as well as the nature of the indicator material and the incubation temperature[4–7].

However, it is not known whether or not these apparent equilibria indeed represent a truly uniform distribution of the indicator material throughout the extracellular compartment. Experiments were designed to verify these assumptions by measuring the distribution of inulin. Tissues exposed to inulin in vivo and in vitro were sectioned into peripheral and central parts and their concentration of radioactivity was compared with each other. With this method a direct estimation of the indicator distribution could be made.

Female golden hamsters of the London School of Hygiene strain inbred at this Institute through over 20 generations, weighing between 100 and 150 g, were used. For the in vivo experiments 8 animals were bilaterally nephrectomized under ether anesthesia and injected in the jugular vein with 0.2 ml (0.68 μc) of 100 mg% solution of inulin-carboxyl-C^{14} (New England Nuclear Corporation, Boston) in phosphate buffer (pH 7.4). Time-course studies demonstrated that the serum inulin level became constant within 60 min. After an additional period of 20 min, a piece of the left ventricle (approximately 100 mg) and the soleus muscle were excised. These tissues were washed free of any superficial radioactive contamination by 6 brief immersions in fresh Ringer's solution, then gently blotted and frozen on dry ice. The frozen tissues were sectioned with a razor blade and divided into 2 groups, a cube-like central part (15–32 mg) and a total of 6 peripheral parts (one for each face of the cube), and the radioactivity content determined separately in the following way: After weighing in tared scintillation vials these tissue pieces were digested with 0.5 ml of a toluene-soluble quarternary ammonium base (NCS, Nuclear Chicago Corporation, Chicago) at 50 °C for 20 h. Fifteen ml of scintillation fluid (4% Liquifluor in toluene, Pilot Chemicals, Watertown, Mass.) were then added to each vial. The radioactivity was measured in a refrigerated scintillation counter (Nuclear Chicago, Model 720), and corrected for background and quenching. In vitro studies were carried out on pieces of left ventricle and intact soleus muscles (cut at the tendon and left attached to the bone). Groups of 6 tissues were incubated for 1, 2, 3 or 5 h at 25 °C in Krebs-Ringer-phosphate buffer, pH 7.1, which contained 25 mg% inulin-carboxyl-C^{14} (85 μc in 100 ml), while being gassed with a mixture of 95% O_2 and 5% CO_2. The distribution of the radioactivity in the center and the surface parts was determined as described above.

The in vivo experiments revealed a nearly uniform distribution of label in the soleus and myocardium. Inside sections of myocardium contained an average of 5% less, those of the soleus muscle an average of 6% more radioactivity than the surrounding tissue. However, in the in vitro experiments no such uniformity was evident. The Figure clearly demonstrates that the central portion of these tissues contained markedly less radioactivity.

Since the pattern of the label distribution is the same in the undamaged soleus as in the myocardial tissue which



Distribution of inulin-carboxyl-C^{14} in tissues incubated in Krebs-Ringer-phosphate buffer (pH 7.1) containing 25 mg% inulin and gassed with a mixture of 95% O_2 and 5% CO_2. Each bar represents the average value of 6 experiments with standard error.

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THE SYNTHESIS OF COLLAGEN BY CHICK BONE RUDIMENTS *IN VITRO*

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RECENT investigations have conclusively demonstrated that in cell culture systems [2, 6, 12, 14, 18] and organ cultures [8, 16] the synthesis of collagen is dependent upon the presence of ascorbic acid. Present evidence suggests that ascorbic acid is concerned in the microsomal hydroxylation of proline in a collagen precursor [9, 13, 21].

The addition of ascorbic acid at a concentration of 50 μg/ml to the synthetic medium in which chick limb-bone rudiments are cultivated, allows collagen synthesis to proceed [8, 16] and also inhibits the excessive hydration of the cartilage [16] that usually occurs during growth in a chemically defined medium [1]; by both biochemical and histological criteria a much more normal extracellular matrix is synthesized in the presence of the vitamin [16]. The present paper records data on the relationships between the level of ascorbic acid in the culture medium and (a) the dry weight/wet weight ratio of the explants, which serves as an index of the degree of hydration, and (b) the collagen content of the rudiments after growth *in vitro*. Experiments have also been made with isoascorbic acid and dehydroascorbic acid to ascertain the specificity of ascorbic acid in collagen synthesis.

A preliminary report of part of this study has been published elsewhere [17].

MATERIALS AND METHODS

Explants.—Paired sets of three bone rudiments, tibiotarsus, femur and humerus from 7-day-old chick embryos were used. The selection of suitable embryos and the isolation of the explants have been described previously [15].

Culture technique.—The culture dishes and conditions of incubation were similar to those described by Fell and Weiss [3] with minor modifications. The dishes were incubated at 38.5°C in a chamber gassed with a mixture composed of 75 per cent N_2: 20 per cent O_2: 5 per cent Co_2. The chemically defined medium was the same as that described previously [16]. For the experiments with ascorbic acid and related com-

[1] Revised version received January 11, 1967.

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pounds, the material was dissolved in the medium immediately before sterilisation by passing through a Millipore filter. The media were dispensed into aliquots sufficient for one medium change only, and stored at −25°C. All media were changed every second day.

L-Ascorbic acid was obtained from British Drug Houses and Sigma, D-isoascorbic acid from Sigma, dehydroascorbic acid from Mann Laboratories and glutathione from British Drug Houses.

Measurement of growth.—The lengths of the explanted rudiments were measured at the beginning and end of each experiment. Wet and dry weights were determined at the end of the culture period by methods previously described [15, 16].

Collagen content.—The total amount of hydroxyproline in sets of rudiments, and in some cases in their culture media, was determined as before [15]. The collagen content was then obtained by multiplying the value for the hydroxyproline content by 7.46 [5].

RESULTS

Effect of different concentrations of ascorbic acid on the hydration of embryonic limb-bones in vitro

Sets of 7-day-old limb-bone rudiments were cultivated for eight days in either chemically defined medium alone or in medium to which ascorbic acid in various concentrations had been added; the levels used ranged from 0 to 225 μg/ml. The dry weights of sets of explants increased with increasing ascorbic acid concentration up to about 50 μg/ml, and at the same time the wet weights decreased (see also [16]). The ratio of dry weight/wet weight of explants treated with different doses of ascorbic acid is shown graphically in Fig. 1, which demonstrates that a dose of ascorbic acid of approximately 50 μg/ml is sufficient to obtain the maximal ratio of dry weight/wet weight of the sets of explants used; this result supports the previous observations at the single dose level of 50 μg/ml [16].

Effect of different concentrations of ascorbic acid on the collagen content of embryonic limb-bones in vitro

The collagen contents of sets of 7-day bone rudiments grown for 8 days in different concentrations of ascorbic acid were calculated from the hydroxyproline contents. Fig. 2 shows that about 80 μg/ml of ascorbic acid is necessary to achieve maximum collagen synthesis in the cultured explants; the maximum percentage of dry weight that is collagen is about 18.5 per cent. Additional experiments have shown that concentrations of ascorbic acid up to 400 μg/ml do not increase further the ratio of collagen/dry weight; no growth inhibition in terms of length or dry weight has been noted at the highest levels used.

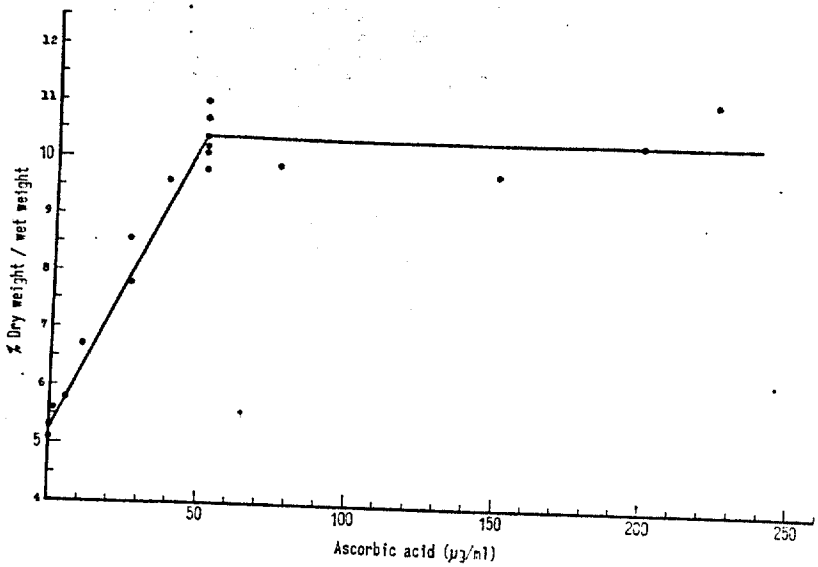

Fig. 1.—Percentage dry weight/wet weight of sets of chick bone rudiments grown for eight d in synthetic medium with varying concentrations of ascorbic acid.

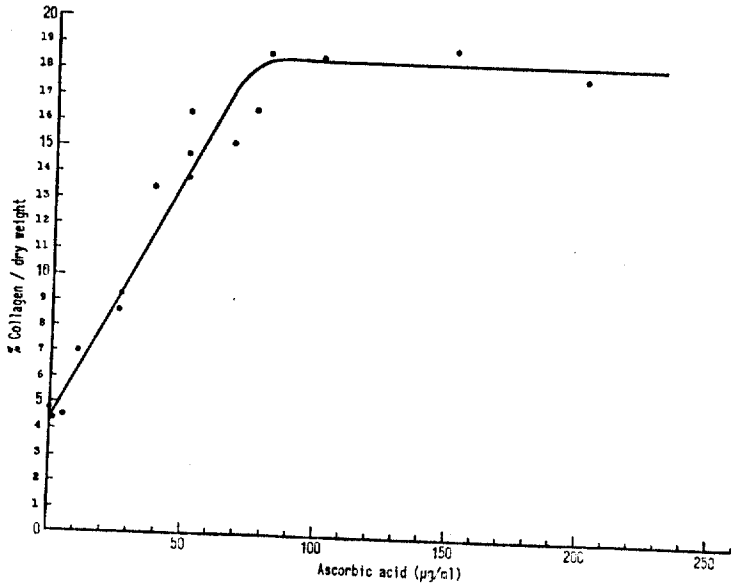

Fig. 2.—Percentage collagen/dry weight of sets of chick bone rudiments grown for eight days i synthetic medium with varying concentrations of ascorbic acid.

Relative ability of compounds related to ascorbic acid to maintain collagen synthesis in vitro

It has been shown previously that after 3–4 days' growth *in vitro* no further collagen synthesis occurs unless ascorbic acid is then added [8, 16]; the same level of collagen synthesis is observed at 8 days whether the vitamin is added on the fourth day and subsequently, or from the beginning of the experiment [16]. Thus if compounds related to ascorbic acid were added after four days' prior growth in synthetic medium alone, their direct action on collagen synthesis could be observed since it would then be unlikely that they were acting to spare the endogenous ascorbic acid. Table I shows the results of such an experiment with isoascorbic acid and dehydroascorbic acid. The values for the dry weight/wet weight ratios and the collagen contents of untreated and ascorbic acid treated controls are similar to previously published results [16]. Isoascorbic acid at a concentration of 50 μg/ml had an appreciable ascorbic acid-like activity, which was about a third of that of an equivalent dose of

TABLE I. *The dry weight, percentage dry weight/wet weight, and hydroxyproline content of sets of chick limb-bone rudiments grown under various conditions with compounds related to ascorbic acid.*

Condition of *in vitro* growth	Dry weight (mg)	Dry weight / Wet weight × 100	OH-proline (μg)	
			Explants	Medium
Synthetic medium for eight days	1.47	5.7	9.7	15.3
	1.66	6.3	11.5	19.4
Synthetic medium with the addition of [illegible] μg/ml ascorbic acid for eight days	1.93	9.6	38.0	27.7
	1.95	10.1	36.2	—[a]
Synthetic medium for four days; then four days with the addition of 50 μg/ml isoascorbic acid	1.85	8.1	20.6	21.3
	2.02	7.3	22.5	23.3
Synthetic medium for four days; then four days with the addition of 100 μg/ml isoascorbic acid	1.68	6.8	16.8	16.9
Synthetic medium for four days; then four days with the addition of 50 μg/ml dehydroascorbic acid	1.43	7.6	10.5	17.2
Synthetic medium for four days; then four days with the addition of 100 μg/ml dehydroascorbic acid	1.37	8.7	13.6	18.6

[a] Medium not analysed.

ascorbic acid; the higher dose of 100 μg/ml was less effective. Dehydroasc bic acid did not appear to have any activity at concentrations of either 50 100 μg/ml.

Table II records additional data showing that dehydroascorbic acid do not allow any increase in collagen synthesis over untreated controls. O

TABLE II. *The dry weight, percentage dry weight/wet weight, and hydroxypr line content, of sets of chick limb-bone rudiments grown in synthetic mediu for eight days with various additions to medium.*

Additions to synthetic medium	Dry weight (mg)	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	OH-proline (μg)
None	1.63	6.6	12.8
50 μg/ml dehydroascorbic acid	0.98[a]	5.2	6.6
	1.06[a]	7.4	12.3
	1.64	7.3	11.4
60 μg/ml freshly prepared dehydroascorbic acid	1.69	6.9	9.3
50 μg/ml dehydroascorbic acid + 60 μg/ml glutathione	1.73	7.7	13.6
75 μg/ml freshly prepared dehydroascorbic acid + 70 μg/ml glutathione	1.64	5.8	9.0

[a] This batch was slightly grown inhibitory, see text.

sample of dehydroascorbic acid tested was somewhat growth inhibitory i terms of length and dry weight; the growth inhibition was probably due t impurities in this sample. Fresh dehydroascorbic acid was prepared by th method of Teruuchi and Mochizuki [20]. Table II also records two expe ments in which dehydroascorbic acid was tested together with glutathion The hydroxyproline contents of sets of explants treated in this way were sim lar to those of untreated controls.

DISCUSSION

It has been shown previously that the addition of ascorbic acid to chemicall defined medium greatly increases the collagen synthesis of cartilaginou limb-bone rudiments grown *in vitro* [8, 16], and also eliminates the excessiv hydration of the cartilage [16]. The results reported in this paper have show

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that a minimum of 50 μg/ml of ascorbic acid is necessary to inhibit completely the excessive hydration. However, about 80 μg/ml of ascorbic acid is necessary for maximum collagen synthesis in this system, even though histologically the cartilage matrix appears normal at 50 μg/ml [16]. It is probable that the high level needed reflects the great instability of the vitamin in tissue culture media [11], rather than actual utilisation by the tissue. It seems very important to ensure that enough ascorbic acid is added to the medium of tissue growing *in vitro*, where collagen synthesis is an integral part of the explant's growth; different media and gas mixtures may alter this requirement.

The results of the experiments with dehydroascorbic acid in place of ascorbic acid demonstrate that the oxidised form of the vitamin is unable to effect any increase in collagen synthesis over untreated controls. It is also significant that the oxidised form of the vitamin does not appear to have any activity in the presence of glutathione. Teruuchi and Mochizuki have shown that *in vivo*, dehydroascorbic acid is quickly reduced by the erythrocytes [20], and Hughes has found that this reaction requires glutathione [7]. These facts together with the experiments detailed in this paper probably indicate that the reduction enzyme is not present in chick limb-bone rudiments; it would be very interesting to know if adult cartilage or bone can perform this reduction, since it appears that the vitamin can more easily penetrate tissues in the oxidised form [10, 20]. Dehydroascorbic acid is fully active *in vivo* [4]. The results in this paper do not fully agree with those reported by Schimizu, McCann and Keech [19], who found that the synthesis of collagen in human dermal fibroblasts in culture, could be stimulated by dehydroascorbic acid provided that cysteine was present; it was less effective, however, than ascorbic acid at the same dose level. The amount of cysteine used by Schimizu *et al.* [19] was far higher than that present in the medium used for this study and it is possible that with very high levels of cysteine or glutathione, dehydroascorbic acid may have some activity. Another possible explanation for the difference between the two sets of results, is that the cell culture system selected cells that are able to perform this reduction, and the fact that the cells used by these workers do not make the tissue-specific chondromucoprotein may be an indication of their altered capabilities. Also of course, the reduction enzyme may be present in dermal cells but not in cartilage or bone.

It has been shown in this paper that isoascorbic acid has an appreciable ascorbic acid-like activity. The activity found is in agreement with the work of Jeffrey and Martin [8] who showed that it was about half as active as ascorbic acid when administered to somewhat older rudiments. Shimizu

et al. [19] have found that isoascorbic acid can completely replace ascorbic acid when used at 200 μg/ml. In the study reported in this paper it was not possible to test isoascorbic acid at higher levels, since the compound was then growth inhibitory. Isoascorbic acid does not appear to have much activity when tested *in vivo* [4, 5].

SUMMARY

The effect of doses of ascorbic acid ranging from 0 to 400 μg/ml of synthetic medium, has been investigated in cultures of 7-day chick limb-bone rudiments grown for eight days. The minimum concentration of ascorbic acid necessary to prevent the excessive hydration usually observed in bone rudiments *in vitro* was 50 μg/ml. A higher level of 80 μg/ml was the minimum dose that would support maximum collagen synthesis in the explants. Dehydroascorbic acid failed to prevent the hydration and did not promote collagen synthesis. Isoascorbic acid at 50 μg/ml was about one-third as active as an equal dose of ascorbic acid in promoting collagen synthesis.

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The figures were prepared by M. Applin.

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Human dermal fibroblasts in monolayer tissue culture. Effect of ascorbic acid analogues

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The effect of some ascorbic acid analogues and two other reducing substances on collagen synthesis, as well as on cell growth and acid mucopolysaccharide production, was studied in monolayer tissue culture of human dermal fibroblasts. D-isoascorbic acid was as effective as L-ascorbic acid in promoting collagen synthesis if the comparison was made at relatively high concentrations such as 200 μg per milliliter of culture medium. Dehydroascorbic acid was ineffective when it was added alone, but was effective when added with cysteine, although inferior to equivalent amounts of ascorbic acid. Neither cysteine nor hydroquinone affected hydroxyproline synthesis. Cysteine seemed to have a sparing effect when it was added with ascorbic acid. It is suggested that the active site of ascorbic acid in hydroxyproline synthesis is its hydroxyradical or radicals and that there is a relative lack of stereospecificity for this vitamin.

Although tissue culture would appear to offer a direct approach to the study of both the physical and chemical properties of connective tissue formed by normal and pathological specimens (including samples from individuals with inherited genetic defects of connective tissue), its usefulness has been impaired by the lack of collagen production by human, as compared with animal or avian, connective tissue cells in vitro. If suitable methods could be found and the conditions for promotion of any one constituent established and understood in biochemical terms, a way would be open for the study of many clinical problems.

A step in this direction was made in a recent report from this laboratory[1] which demonstrated that human dermal fibroblasts grown in monolayer culture on 83 per cent Eagle's basal medium, 15 per cent heat-deactivated human serum obtained from healthy donors, and 2 per cent embryo extract, will synthesize collagen provided the medium is supplemented with ascorbic acid. The actual role of ascorbic acid in collagen synthesis is still uncertain although the bulk of

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the evidence would suggest that the conversion of proline to hydroxyproline is involved. Furthermore, it is not known whether ascorbic acid acts as an obligatory coenzyme or whether other reducing agents can be substituted for it. Consequently, we studied the effect of a number of ascorbic acid analogues, as well as two other reducing agents, on our tissue culture system.

Materials and methods

Primary cell cultures were grown, with one exception, from normal dermis obtained from surgical patients. The age and sex of donors and the anatomical skin sites are summarized in Table I. The method of sample preparation and culture has been reported in detail previously.[1] Cells were harvested and counted in a hemocytometer. Proline and hydroxyproline in the cell fraction were determined by a modified Neuman-Logan method[3] and Messer's method,[4] respectively, after hydrolysis with 6N HCl in an autoclave at 15 pounds for 3 hours (Table II). The total acid mucopolysaccharides in the medium were measured by a modification of Castor and Prince's method.[5] The acid mucopolysaccharide content of uninoculated medium was determined in every batch, and this value was subtracted to give a net synthesis of acid mucopolysaccharides by the cells during the incubation period. In some instances, the acid mucopolysaccharide fractions from pooled, used medium were further purified and fractioned.[6]

Experimental plan

All cultures in a given experiment were started from 10^5 cells per bottle. Stock cultures were transferred every 14 days and fed with control medium throughout the experiment. Each bottle was planted from a pooled suspension of freshly harvested fibroblasts, prepared from stock cultures that were identical with respect to number of transfers and previous handling. Cells were allowed to attach to the glass, fed fresh medium every 2 to 3 days, and cultured until they reached a maximum cell density, usually 14 days after transfer. At this time, 5 bottles were harvested, the cells counted, and their proline and hydroxyproline content

Table I. Origin of skin samples

Cell line	*Age*	*Sex*	*Anatomical site*
H. C.	7	M	Back of ears
A. B.	12	M	Left thigh
F. T.	38	F	Inguinal hernia sac
E. T.	41	F	Abdominal wall
T. K.	49	F	Abdominal wall
G. O.*	51	M	Right thigh
P. S.	60	F	Abdominal wall
H. G.	90	M	Hernia sac

*Patient suffered from generalized scleromyxedema.

Table II. Autoclaving time and yields of proline and hydroxyproline from cell fractions

Autoclaving time (Hr.)	*Proline (μg)*	*Hydroxyproline (μg)*
3	112.3 ± 10.81	15.2 ± 2.81
6	96.5 ± 6.24	13.9 ± 1.61
9	79.3 ± 4.11	6.9 ± 1.73

Cell fractions were autoclaved with 6N HCl at 15 pounds.

Table III. Effects of D-isoascorbic acid on acid mucopolysaccharide and collagen synthesis by human dermal fibroblasts in tissue culture

Donor	Transfers	Days after explant	Medium supplement (μg/ml.)		Cell population (10^3/bottle)	Rate of synthesis: AMPS-uronate (10^{-12}Gm./cell/day)	Proline (μM/10^7 cells/day)	Hydroxyproline (μM/10^7 cells/day)
H. C.	3	84	A.A.	50	1,415 ± 109.4	22.1 ± 2.64	0.49 ± 0.167	0.10 ± 0.079
			D-iso-A.A.	50	1,416 ± 125.0	23.7 ± 2.19	0.41 ± 0.078	0.06 ± 0.023
			D-iso-A.A.	200	1,461 ± 121.7	26.7 ± 2.69	0.60 ± 0.133	0.16 ± 0.085
F. T.	3	128	A.A.	50	699 ± 27.3	27.8 ± 6.11	0.38 ± 0.117	0.08 ± 0.002
			D-iso-A.A.	50	835 ± 90.6	29.4 ± 6.45	0.31 ± 0.038	0.02 ± 0.007
			D-iso-A.A.	200	724 ± 63.8	26.9 ± 3.31	0.38 ± 0.065	0.09 ± 0.025
E. T.	1	49	A.A.	200	850 ± 70.2	31.1 ± 5.36	0.65 ± 0.190	0.21 ± 0.073
			D-iso-A.A.	50	1,141 ± 119.2	26.3 ± 2.07	0.40 ± 0.074	0.06 ± 0.022
			D-iso-A.A.	200	1,319 ± 283.0	24.7 ± 7.57	0.73 ± 0.260	0.24 ± 0.076
P. S.	1	46	A.A.	200	1,893 ± 119.6	17.1 ± 5.08	0.41 ± 0.039	0.06 ± 0.018
			D-iso-A.A.	50	1,710 ± 220.1	33.3 ± 3.31	0.50 ± 0.069	0.06 ± 0.016
			D-iso-A.A.	200	1,502 ± 197.2	35.9 ± 9.01	0.86 ± 0.265	0.13 ± 0.053
10 Cell lines (controls)			None		Average (range)	20.3 (10.2 – 35.0)	0.19 (0.05 – 0.31)	0.006 (0 – 0.01)
8 Experiments with 6 cell lines			A. A.	200	Average (range)	20.6 (7.6 – 31.1)	0.49 (0.30 – 0.74)	0.15 (0.06 – 0.24)

A. A., Ascorbic acid; AMPS, acid mucopolysaccaride; D-iso-A. A., D-isoascorbic acid.
All values are mean ± S.D. from at least 4 bottles.

assayed. After this preliminary period, the bottles were divided into control and experimental groups so that each group comprised at least 6 bottles. For the following week, the medium fed to the experimental groups was supplemented with whatever chemical was to be tested. At the end of the culture cycle, usually 21 days after transfer, the cells were harvested and counted, and the proline and hydroxyproline content determined for the "cell fraction" of each bottle. Acid mucopolysaccharides were determined in the medium. For this purpose the medium was changed completely 24 hours prior to the harvesting.

Ascorbic acid, D-isoascorbic acid,* dehydroascorbic acid,† and cysteine hydrochloride were dissolved in distilled water and filtered through a Millipore membrane for sterilization. Hydroquinone was dissolved in absolute alcohol, filtered for sterilization, and diluted with aqueous cysteine hydrochloride solution. These solutions were kept frozen and added to the medium immediately before feeding at the rate of 1.0 ml. for every 200 ml. of medium. The pH of the medium was adjusted to 7.4 with 0.3N NaOH.

Results

The results have been summarized in Tables III to VI. Collagen synthesis has been measured in terms of hydroxyproline synthesis, and acid mucopolysaccharide synthesis expressed as uronic acid. The average rate of hydroxyproline synthesis by human dermal fibroblasts in monolayer tissue cultures in control

*Nutritional Biochemical Corporation, Cleveland, Ohio.
†K & K Laboratory, Inc., Plainview, N. Y.

Table IV. Effects of dehydroascorbic acid on acid mucopolysaccharide and collagen synthesis by human dermal fibroblasts in tissue culture

Donor	Transfers	Days after explant	Medium supplement (μg/ml.)	Cell population 10^3/bottle	Rate of synthesis: AMPS-uronate 10^{-12} Gm./cell/day	Rate of synthesis: Proline (μM/10^7 cells/day)	Rate of synthesis: Hydroxyproline (μM/10^7 cells/day)
T. K.	1	47	A.A. 200	1,772 ± 207.9	17.1 ± 4.21	0.43 ± .177	0.20 ± .107
			Dehydro-A.A. 200	1,760 ± 69.2	13.1 ± 1.78	0.08 ± .024	0.01 ± .006
			Dehydro-A.A. 200, Cysteine-HCl 260	2,250 ± 145.9	15.0 ± 4.47	0.27 ± .037	0.06 ± .005
P. S.	2	53	A.A. 200	1,106 ± 181.4	24.1 ± 6.24	0.49 ± .057	0.11 ± .030
			Dehydro-A.A. 200	1,512 ± 87.5	21.9 ± 5.59	0.24 ± .037	0.02 ± .005
			Dehydro-A.A. 200, Cysteine-HCl 260	1,466 ± 179.9	23.3 ± 1.99	0.33 ± .097	0.07 ± .017
G. O.	1	40	A.A. 200	2,190 ± 137.3	7.6 ± 0.89	0.30 ± .061	0.06 ± .015
			Dehydro-A.A. 200	2,544 ± 210.7	8.7 ± 1.61	0.20 ± .114	0.01 ± .007
			Dehydro-A.A. 200, Cysteine-HCl 260	3,934 ± 340.1	7.0 ± 0.37	0.18 ± .058	0.01 ± .009
10 Cell lines (controls)			None	Average (range)	20.3 (10.2 – 35.0)	0.19 (0.05 – 0.31)	0.006 (0 – 0.01)
8 Experiments with 6 cell lines			A. A. 200	Average (range)	20.6 (7.6 – 31.1)	0.49 (0.30 – 0.74)	0.15 (0.06 – 0.24)

A. A., Ascorbic acid; AMPS, acid mucopolysaccharide; dehydro-A. A., dehydroascorbic acid. All values are mean ± S.D. from at least 4 bottles.

medium was 0.006 μM per 10^7 cells per day, whereas the addition of 200 μg of ascorbic acid elevated this average value to 0.15 μM per 10^7 cells per day.

Effect of D-*isoascorbic acid.* With the addition of 50 μg of D-isoascorbic acid per milliliter of medium, there was a small but definite increase of hydroxyproline synthesis above the control level. This synthesis was, however, smaller than that in the group supplemented with 50 μg of L-ascorbic acid. On the other hand, when 200 μg of D-isoascorbic acid was added per milliliter of medium, the cells synthesized hydroxyproline at about the same rate as in a 200 μg L-ascorbic acid-supplemented group. Some variations from experiment to experiment will occur in this type of work. Thus, the response shown by line P.S. in Table III appears abnormal because 0.06 μM of hydroxyproline is an unusually low response to the addition of 200 μg of ascorbic acid per milliliter of medium, especially if one considers the hydroxyproline synthesis obtained with 200 μg of D-isoascorbic acid. Up to the concentrations of 200 μg per milliliter of medium, D-isoascorbic acid showed no toxicity on the cells, as judged by either the cell count or the morphology under the light microscope. There was no significant difference in the rate of acid mucopolysaccharide synthesis between the D-isoascorbic and L-ascorbic acid groups (Table III), or in response to variation in dosage of the supplement.

Table V. Effects of cysteine and reduced diphosphopyridine nucleotide on acid mucopolysaccharide and collagen synthesis by human dermal fibroblasts in tissue culture

Donor	Transfers	Days after explant	Medium supplement (μg/ml.)		Cell population (10^3/bottle)	Rate of synthesis: AMPS-uronate (10^{-12} Gm./cell/day)	Proline (μM/10^7 cells/day)	Hydroxyproline (μM/10^7 cells/day)
H.C.	2	70	A. A.	200	1,981 ± 219.6	19.1 ± 2.62	0.45 ± .225	0.13 ± .086
			Cysteine-HCl	260	2,002 ± 159.4	17.5 ± 2.21	0.23 ± .066	0.00 ± .000
			Cysteine-HCl A. A.	260 200	2,172 ± 129.2	21.5 ± 1.82	0.53 ± .199	0.14 ± .074
F.T.	2	107	A. A.	200	1,368 ± 379.2	26.3 ± 6.46	0.42 ± .116	0.18 ± .029
			Cysteine-HCl	260	2,679 ± 363.5	13.5 ± 1.71	0.23 ± .007	0.00 ± .000
			Cysteine-HCl A. A.	260 200	1,413 ± 145.5	28.5 ± 6.83	0.93 ± .126	0.40 ± .079
T.K.	3	71	A. A.	200	1,737 ± 99.2	22.6 ± 4.47	0.74 ± .130	0.24 ± .064
			Cysteine-HCl	260	2,973 ± 250.4	14.7 ± 0.99	0.37 ± .023	0.01 ± .000
			Cysteine-HCl A. A.	260 200	1,950 ± 288.1	19.4 ± 6.04	0.88 ± .209	0.30 ± .094
10 Cell lines (controls)			None		Average (range)	20.3 (10.2 – 35.0)	0.19 (0.05 – 0.31)	0.006 (0 – 0.01)
8 Experiments with 6 cell lines			A. A.	200	Average (range)	20.6 (7.6 – 31.1)	0.49 (0.30 – 0.74)	0.15 (0.06 – 0.24)

A. A., Ascorbic acid; AMPS, acid mucopolysaccharide.
All values are mean ± S.D. from at least 4 bottles.

Effect of dehydroascorbic acid. The addition of 200 μg of dehydroascorbic acid per milliliter of medium did not appear to have any significant effect on the hydroxyproline synthesis of our system. When dehydroascorbic acid was added with cysteine hydrochloride, however, the cells synthesized a measurable amount of hydroxyproline, which was less than that synthesized under influence of a comparable amount of L-ascorbic acid. Again, there was no significant difference in the rate of acid mucopolysaccharide synthesis among the three groups (Table IV). The response of cell line G. O., which was the only line derived from an individual suffering from a systemic disease involving skin, is discussed below.

Effect of cysteine and hydroquinone. As shown in Tables V and VI, additions of either cysteine hydrochloride or hydroquinone, 260 μg and 10 μg per milliliter of medium, respectively, did not affect the hydroxyproline synthesis of human dermal fibroblasts in our system. Cysteine HCl was able to enhance collagen synthesis obtained with 200 μg per milliliter of ascorbic acid. In addition, the rate of general protein synthesis, as judged by proline incorporation into the cell fraction, is increased beyond that expected from ascorbic acid supplementation alone, when the vitamin was added together with cysteine. The rate of proline incorporation was lowest in groups to which cysteine alone was added and highest in cysteine plus ascorbic acid groups. Differences in the rate of

Table VI. Effects of hydroquinone on acid mucopolysaccharide and collagen synthesis by human dermal fibroblasts in tissue culture

Donor	Transfers	Days after explant	Medium supplement (μg/ml.)	Cell population (10^3/bottle)	AMPS-uronate (10^{-12} Gm./cell/day)	Rate of synthesis: Proline (μM/10^7 cells/day)	Rate of synthesis: Hydroxyproline (μM/10^7 cells/day)
H. G	2	73	A. A. 200 Cysteine-HCl 260	938 ± 150.8	39.3 ± 1.19	0.97 ± .093	0.31 ± .022
			Cysteine-HCl 260 Hydroquinone 10	1,004 ± 102.1	27.4 ± 4.43	0.35 ± .024	0.01
			A. A. 200 Cysteine-HCl 260 Hydroquinone 10	833 ± 119.3	38.6 ± 7.15	0.71 ± .044	0.20 ± .019
A. B.	2	69	A. A. 200 Cysteine-HCl 260	2,129 ± 189.2	15.7 ± 6.00	0.36 ± .059	0.11 ± .038
			Cysteine-HCl 260 Hydroquinone 10	2,748 ± 94.5	13.5 ± 0.73	0.15 ± .021	0.00
			A. A. 200 Cysteine-HCl 260 Hydroquinone 10	1,438 ± 203.5	26.1 ± 5.51	0.31 ± .029	0.07 ± .018
10 Cell lines (controls)			None	Average (range)	20.3 (10.2 – 35.0)	0.19 (0.05 – 0.31)	0.006 (0 – 0.01)
8 Experiments with 6 cell lines			A. A. 200	Average (range)	20.6 (7.6 – 31.1)	0.49 (0.30 – 0.74)	0.15 (0.06 – 0.24)

A. A., Ascorbic acid; AMPS, acid mucopolysaccharide.
All values are mean ± S.D. from at least 4 bottles.

proline concentration among these three groups did not necessarily correspond to that of hydroxyproline synthesis, which suggests that ascorbic acid is involved not only in hydroxyproline synthesis, but also activates other cellular protein synthesis. As described earlier,[1] when ascorbic acid was added after the cells had reached their maximal density, cell populations tended to decrease. Although additions of cysteine seemed to increase the maximum density of cells, they could not restore this decreasing trend produced by ascorbic acid additions (Tables IV and V). The rate of acid mucopolysaccharide synthesis was significantly lower in cysteine groups as compared to those in which ascorbic acid was added. As reported previously, however, the rate of acid mucopolysaccharide synthesis generally decreased with increasing cell populations.[1] Thus, it seems that the effect of cysteine on the rate of acid mucopolysaccharide synthesis is rather indirect, that is, its primary effect is on the cell population.

Increases of medium acid mucopolysaccharide after incubation with the cells were found only in the nonsulfated acid mucopolysaccharide fractions. Increase

of sulfated acid mucopolysaccharide could not be detected with the analytical tools used in the present study.

Discussion

Robertson[7] reported that the impaired collagen synthesis in scorbutic guinea pigs was completely rectified by large doses of D-ascorbic acid given frequently enough to maintain its concentration above 200 μg per gram of granulation tissue. Gould,[8] using a paired sponge biopsy technique in scorbutic guinea pigs, found that D-isoascorbic acid had no direct action on collagen synthesis, and assumed that this compound may perhaps be unable to cross fibroblast membrane barriers. The results presented here strongly suggest that D-isoascorbic acid can not only be transported across the cell membranes of fibroblasts, but can increase collagen synthesis. At higher concentrations, e.g., 200 μg per milliliter of culture medium, this compound is as effective as L-ascorbic acid with respect to hydroxyproline synthesis by human dermal fibroblasts in tissue culture. On the basis of this relative lack of stereospecificity, Robertson[7] excludes ascorbic acid from the class of compounds defined as coenzymes.

Dehydroascorbic acid has been reported[8, 9] to be as effective as ascorbic acid on collagen synthesis in scorbutic animals when administered in vivo. Teruuchi and Mochizuki[10] reported that human erythrocytes are capable of reducing dehydroascorbic acid to ascorbic acid in vitro. Martin[9] administered small doses of ascorbic acid-C^{14} and dehydroascorbic acid-C^{14} to rats and found that the ascorbic acid in the brain and eye approached equilibrium with serum more rapidly when the oxidized form of the vitamin was given. Since the reduced form of the vitamin is a relatively strong acid, whereas the oxidized form is nonionic and more lipophilic, Martin[9] suggested that dehydroascorbic acid would be expected to cross the cellular membrane more rapidly than ascorbic acid. Since no appreciable amount of hydroxyproline could be detected when dehydroascorbic acid was added alone, it was assumed that hydroxyproline synthesis requires a structure involving the reduced hydroxyradical or radicals of ascorbic acid. The current work does not determine whether the reduction occurs inside or outside the cells. At any rate, human dermal fibroblasts appeared to be incapable of reducing dehydroascorbic acid to ascorbic acid under our "control" conditions.

It has been reported[11] that ascorbic acid can be replaced by hydroquinone in the conversion of p-hydroxyphenylpyruvate to homogentisate. The role of ascorbic acid in tyrosine metabolism has proved to be a rather unusual one for a vitamin, that is, ascorbic acid has the ability to protect an enzyme, p-hydroxyphenylpyruvic acid oxidase, from inhibition by its substrate. Since hydroquinone did not show any effect on collagen synthesis in our system, it may be suggested that ascorbic acid acts on collagen synthesis in a different way.

The cell line G. O. was derived from a patient with generalized scleromyxedema, which is a rare disease included in the category of lichen myxedematosus. This diagnosis was confirmed by skin biopsy and a complete necropsy and will be reported in full elsewhere (Dr. H. Pinkus). During life, histological and chemical tests revealed the accumulation of enormous amounts of acid mucopolysaccharide in the skin and a marked elevation of both hyaluronic acid and

sulfated acid mucopolysaccharide levels in the serum. In tissue culture, however, no abnormal accumulation of acid mucopolysaccharide within the dermal fibroblasts was found in the primary explant cultures. In the first transfer, 40 days after explantation, both acid mucopolysaccharide and collagen synthesis were rather lower than those of the cell lines derived from healthy subjects (Table IV), but thereafter both these values were within the normal range.

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Tyrosyluria Resulting from Inhibition of *p*-Hydroxyphenylpyruvic Acid Oxidase in Vitamin C-deficient Guinea Pigs

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Tyrosyluria, the excretion of *p*-hydroxyphenyl metabolites of tyrosine, such as *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid, occurs after the administration of tyrosine to scorbutic guinea pigs (1, 2), scorbutic man (3, 4), and to premature infants (5). A persistent tyrosyluria consisting mainly of *p*-hydroxyphenylpyruvic acid was also present in the unique case of tyrosinosis reported by Medes (6).

Earlier studies *in vitro* have shown that *p*-hydroxyphenylpyruvic acid oxidase has an unusual susceptibility to inhibition by high concentrations of its substrate (7–9). Recently it has been demonstrated that *p*-hydroxyphenylpyruvic acid injected intraperitoneally inhibits *p*-hydroxyphenylpyruvic acid oxidase in scorbutic guinea pigs. In contrast, animals which received adequate amounts of vitamin C were completely protected from this inhibition (10).

In view of these findings, it was of interest to determine whether the tyrosyluria subsequent to the feeding of large amounts of tyrosine to vitamin C-deficient animals was due to an inhibition of this oxidase through the formation of the corresponding α-keto acid.

EXPERIMENTAL PROCEDURE

Materials—*p*-Hydroxyphenylpyruvic acid was obtained from the H. M. Chemical Company, Ltd. 2,6-Dichlorophenolindophenol was purchased from Eastman Organic Chemicals Department, Eastman Kodak Company. Glutathione, L-tyrosine, α-ketoglutaric acid, pyridoxal-5-phosphate, and D-glucoascorbic acid were commercial preparations from the Nutritional Biochemicals Corporation. D-Isoascorbic acid was purchased from Hoffmann-La Roche, Inc. L-Ascorbic acid was obtained from Merck and Company, Inc. Homogentisic acid was purchased from the Cyclo Chemical Corporation. DL-*p*-Hydroxyphenyllactic acid was generously supplied by Dr. H. J. Cahnmann.

Animals—Male, albino guinea pigs, weighing approximately 200 g, were placed on a vitamin C-free diet prepared as described by Woodruff *et al.* (11).[1] Control groups were given the same diet supplemented orally with 25 mg per day of L-ascorbic acid. After 1 week, the animals on the vitamin C-free diet were still gaining weight and showed no signs of scurvy, and the concentration of ascorbic acid in the liver was approximately 3.0 mg/100 g wet weight of liver. Animals on the vitamin C-free diet for 2 weeks developed the typical signs of scurvy: weight loss, swollen joints, and hemorrhages in the knee joints. At this time the concentration of ascorbic acid in the liver had fallen to less than 1.0 mg/100 g wet weight of liver.

Preparation of Liver Homogenate—At the time of death, the livers were removed and a 33% homogenate was prepared. Liver (5 g) was homogenized with 10 ml of 0.2 M sodium phosphate buffer, pH 6.5, in a Potter-Elvehjem type glass homogenizer at 5°. The homogenate was centrifuged at 10,000 × *g* for 10 minutes and the resulting supernatant fraction was used in the experiments described below. Homogenates of kidney were prepared in a similar way.

Liver Ascorbic Acid Determination—Liver (1 g) was homogenized with 12.5 ml of 4% trichloroacetic acid at 5°. After centrifugation, suitable aliquots of the deproteinized supernatant fraction were taken for the determination of ascorbic acid according to the method of Roe *et al.* (12).

Determination of Tyrosine and p-Hydroxyphenylpyruvic Acid in Plasma and Urine—Plasma from heparinized blood samples was deproteinized by the addition of an equal volume of 7% perchloric acid. The protein-free, supernatant fraction was neutralized with 14% KOH and the resulting perchlorate removed by centrifugation. The supernatant fraction was then analyzed for tyrosine by the method of La Du and Michael (13) and for *p*-hydroxyphenylpyruvic acid by the same method with the snake venom L-amino acid oxidase omitted. Dilute neutralized urine was analyzed for tyrosine and *p*-hydroxyphenylpyruvic acid directly.

Determination of p-Hydroxyphenyllactic Acid in Urine—Acidified urine was extracted twice with 10 times its volume of acidified, washed, peroxide-free ether. The pooled ether extracts were evaporated at room temperature with a stream of air in the presence of 1.0 ml of 0.2 M sodium phosphate buffer, pH 6.5. After the ether had been completely removed, the residual material was made up to either 2 or 5 ml with the phosphate buffer. The content of *p*-hydroxyphenyllactic acid in the extracts was determined manometrically in the presence of yeast lactic acid dehydrogenase (14) with methylene blue as the hydrogen acceptor. The main compartment of the Warburg vessels contained 1.0 ml of yeast lactic acid dehydrogenase, 5 μmoles of methylene blue, and 0.2 M sodium phosphate buffer, pH 6.5, to make a total volume of 2.0 ml. The side arm contained aliquots

[1] Obtained from Nutritional Biochemicals Corporation.

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TABLE I

Effect of feeding tyrosine on enzymes in normal and scorbutic guinea pigs

Assay conditions. *Tyrosine transaminase*—The reaction was measured spectrophotometrically at 25° by following the appearance of the enol-borate complex of *p*-hydroxyphenylpyruvate at 310 mμ (16). *p-Hydroxyphenylpyruvate oxidase and homogentisic acid oxidase*—The spectrophotometric assay for *p*-hydroxyphenylpyruvic acid oxidase activity was carried out at 25° and the manometric assay of homogentisic acid oxidase activity at 37°, as previously described (10).

Enzyme assay[a]	Normal animals[b]		Scorbutic animals[b]	
	Untreated (6)[c]	Treated (6)	Untreated (10)	Treated (10)
	μmoles of substrate oxidized/hr/g fresh liver[d]			
Liver				
Tyrosine transaminase	17.9 ± 6.9	23.6 ± 11.4	39.5 ± 6.5	86.9 ± 15.3
p-Hydroxyphenylpyruvic acid oxidase	30.4 ± 6.1	24.8 ± 5.1	17.1 ± 2.6	2.6 ± 0.9
Homogentisic acid oxidase	127 ± 22.4	152 ± 20.5	69.5 ± 12.0	101.9 ± 19.7
Kidney				
p-Hydroxyphenylpyruvic acid oxidase	6.6 ± 0.9	5.3 ± 1.7	6.8 ± 1.8	1.1 ± 0.1

[a] Enzyme activities are based on initial rate of oxidation. Transaminase activity was calculated after the initial lag period.
[b] Ascorbic acid levels: normal liver, 5.47 ± 1.3 (mean, ± standard error) mg per 100 g wet weight of liver; scorbutic liver, 1.01 ± 0.47.
[c] Numbers in parentheses = number of animals.
[d] Standard error of the mean is given.

of the urine extracts or standard solutions of *p*-hydroxyphenyllactic acid. The reaction was run at 37° with air as the gas phase, and oxidation was complete within 20 minutes.

The product of this reaction was identified as *p*-hydroxyphenylpyruvic acid and was measured manometrically (7), with purified *p*-hydroxyphenylpyruvic acid oxidase or by spectrophotometric determination of the enol-borate complex (15).

Enzyme Assay Methods—Tyrosine transaminase activity was estimated spectrophotometrically according to the method of Lin and Knox (16). The homogenate was supplemented with α-ketoglutaric acid and pyridoxal-5-phosphate in order to insure optimal activity. *p*-Hydroxyphenylpyruvic acid oxidase was determined spectrophotometrically, and homogentisic acid oxidase was determined manometrically as previously described (9, 10, 17).

RESULTS

Effect of Feeding L-Tyrosine on Tyrosine Oxidation Enzymes in Scorbutic Guinea Pigs—Guinea pigs on the vitamin C-free diet for 2 weeks with typical signs of scurvy, and animals on the same diet supplemented with vitamin C, were divided into control and experimental groups of 6 to 10 animals. The experimental animals were given a total of 400 mg of tyrosine suspended in 4 ml of water (sweetened with sucrose) by feeding 100-mg portions at hourly intervals for 4 hours. One hour after the last dose of tyrosine, the animals were killed and the liver and kidneys rapidly removed and chilled. Homogenates were prepared and used for enzymatic assays as described under the section on "Enzyme Assay Methods." The activities of tyrosine transaminase, *p*-hydroxyphenylpyruvic acid oxidase, homogentisic acid oxidase in liver, and *p*-hydroxyphenylpyruvic acid oxidase in kidney are given in Table I. It was found that feeding tyrosine to normal guinea pigs had no effect on these enzymes. However, there was a marked decrease, over 80%, in the activity of *p*-hydroxyphenylpyruvic acid oxidase in the liver and kidney of scorbutic animals fed tyrosine. The latter group also showed a significant increase in the activity of tyrosine transaminase. *p*-Hydroxyphenylpyruvic acid oxidase was also lower in the scorbutic animals not given tyrosine than in the corresponding control group.

The amount of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid excreted during the 5-hour experimental period was determined, and the degree of tyrosyluria was proportional to the level of tyrosine in the plasma (Table II). It is of interest that at the time of death there was no detectable *p*-hydroxyphenylpyruvic acid in the blood, even though the major tyrosyl metabolite in the urine was *p*-hydroxyphenylpyruvic acid. The urine also contained small amounts of *p*-hydroxyphenyllactic acid, but no detectable tyrosine. This elevation of the amino acid in the blood and the excretion of the corresponding α-keto acid resembles the biochemical findings in phenylketonuria, in which phenylalanine is elevated in blood and phenylpyruvic acid is excreted in the urine.

TABLE II

Tyrosyluria after feeding tyrosine to normal and scorbutic guinea pigs

The estimations of plasma tyrosine, urine *p*-hydroxyphenylpyruvic acid, and urine *p*-hydroxyphenyllactic acid were performed as described under the experimental section. Average values are given.

	Normal animals		Scorbutic animals	
	Untreated (6)*	Treated (6)	Untreated (10)	Treated (10)
	mg per 100 ml of plasma			
Plasma				
Tyrosine	0.5	12	1.0	73
	mg excreted in 5 hrs			
Urine				
p-Hydroxyphenylpyruvate	<0.1	0.6	<0.1	38
p-Hydroxyphenyllactate	<0.1	<0.1	<0.1	0.4

* Numbers in parentheses = number of animals.

Effect of Repeated Feeding of Tyrosine to Vitamin C-deficient Guinea Pigs—Further experiments were undertaken to determine whether guinea pigs less deficient in vitamin C, *i.e.* not frankly scorbutic, would also be susceptible to inhibition of *p*-hydroxyphenylpyruvic acid oxidase by feeding tyrosine. It was also desirable to determine whether inhibition of *p*-hydroxyphenylpyruvic acid oxidase, once induced, might be maintained by repeated small oral supplements of tyrosine. Several groups of guinea pigs were placed on the vitamin C-free diet for 1 week. At this time they were fed 400 mg of tyrosine in 100-mg portions at hourly intervals. Some of the animals were killed 1 hour after the fourth dose of tyrosine, and in all the liver *p*-hydroxyphenylpyruvic acid oxidase activity was found to be inhibited over 90% (Table III). The remaining animals were continued on the vitamin C-free diet and were given 100 mg of tyrosine orally every 12 hours for the next 3 days. Groups of animals were then killed at 1 hour, 4 hours, and 12 hours after the last dose of tyrosine, and the activities of tyrosine transaminase and *p*-hydroxyphenylpyruvic acid oxidase were determined (Table III). Although *p*-hydroxyphenylpyruvic acid oxidase was depressed to about one half of its normal value for several hours, it had returned to its previous normal value by 12 hours. The recovery of *p*-hydroxyphenylpyruvic acid oxidase may represent newly synthesized enzyme or the reactivation of previously inhibited enzyme. It is of interest that under these experimental conditions, the level of tyrosine transaminase remained elevated to about 5 times its value in control animals.

Ability of Various Compounds to Prevent Inhibition of p-Hydroxyphenylpyruvic Acid Oxidase In Vivo—Earlier studies on the inhibition of *p*-hydroxyphenylpyruvic acid oxidase by excess substrate *in vitro* have shown that ascorbic acid is not specifically required to prevent this unusual type of enzyme inhibition and that a number of other reducing agents are also effective. Among these compounds are D-ascorbic acid, D-isoascorbic acid, and 2,6-dichlorophenolindophenol (18, 19). It was therefore of interest to test the effectiveness of some of these compounds *in vivo* in preventing inhibition of *p*-hydroxyphenylpyruvic acid oxidase in vitamin C-deficient guinea pigs fed tyrosine. Groups of guinea pigs were placed on the vitamin C-free diet for 1 week and then given 400 mg of tyrosine in 100-mg portions at hourly intervals. The test compounds were given intraperitoneally, 10 mg one half hour before the first tyrosine feeding, and 10 mg more 2 hours later. Animals were killed 1 hour after the last tyrosine dose and the *p*-hydroxyphenylpyruvic acid oxidase activity of the liver was determined. Plasma levels of tyrosine, *p*-hydroxyphenylpyruvic acid, and liver ascorbic acid were measured at the time of death (Table IV). The results indicated that 2,6-dichlorophenolindophenol and D-isoascorbic acid were able to completely prevent any inhibition of *p*-hydroxyphenylpyruvic acid oxidase, and glucoascorbic acid gave partial protection of the enzyme. The concentration of tyrosine in the plasma was elevated in the groups with inhibited *p*-hydroxyphenylpyruvic acid oxidase and was proportional to the degree of inhibition observed. The concentration of ascorbic acid in the liver indicated that there was a greater retention of D-isoascorbic acid than glucoascorbic acid in this tissue and this finding was in agreement with their relative ability to protect *p*-hydroxyphenylpyruvic acid oxidase.

From these results one would predict that other compounds which have been found to be effective in preventing inhibition of *p*-hydroxyphenylpyruvic acid oxidase by excess substrate *in vitro* would be able to prevent inhibition of this enzyme *in vivo*. However, the effectiveness actually observed *in vivo* will also be modified by their physiological distribution and retention within the liver.

The ability of 2,6-dichlorophenolindophenol to protect the

TABLE III

Effect of prolonged feeding of L-tyrosine on liver p-hydroxyphenylpyruvic acid oxidase and tyrosine transaminase in vitamin C-deficient guinea pigs

Treatment with tyrosine*		Time of death; hrs after last dose of tyrosine	Liver enzyme activities†	
100 mg each hr for 4 hrs	Followed by 100 mg twice daily for 3 days		*p*-Hydroxyphenylpyruvate oxidase	Tyrosine transaminase
			μmoles of substrate oxidized/hr/g fresh liver	
−	−	−	29	13
+	−	1	3	24
+	+	1	12	59
+	+	4	11	55
+	+	12	26	63

* The regimen followed in administering tyrosine orally is described in the text.

† Assays for tyrosine transaminase and *p*-hydroxyphenylpyruvic acid oxidase were as described under Table I. The values given represent the average of 4 animals in each group.

TABLE IV

Protection of p-hydroxyphenylpyruvic acid oxidase in vivo by various compounds

The animals were fed 100 mg of L-tyrosine each hour for 4 hours and killed 1 hour after the last dose of tyrosine. The compounds tested were injected intraperitoneally as follows: 10 mg, 30 minutes before the first tyrosine feeding, and 10 mg more 2 hours later. *p*-Hydroxyphenylpyruvic acid oxidase activity was assayed as described in Table I. Plasma tyrosine, plasma *p*-hydroxyphenylpyruvic acid, and liver ascorbic acid were determined as described in the "Experimental Procedure" section. Average values are given.

Compound	Liver *p*-hydroxyphenylpyruvic acid oxidase	Plasma tyrosine	Plasma *p*-hydroxyphenylpyruvic acid	Liver "ascorbic acid"*
	μmoles of substrate oxidized/hr/g fresh liver	*mg per 100 ml plasma*		*mg per 100 g wet weight tissue*
None (6)†	1.6	50.3	5.6	3.1
L-Ascorbic acid (4)	32.2	12.5	<0.4	28.9
D-Isoascorbic acid (4)	31.0	13.2	<0.1	13.3
D-Glucoascorbic acid (4)	9.5	24.5	0.8	4.5
2,6-Dichlorophenolindophenol (oxidized) (5)	29.0	10.2	<0.1	2.6
2,6-Dichlorophenolindophenol (reduced) (4)‡	29.5	5.9	<0.1	3.3

* D-Isoascorbic acid and D-glucoascorbic react in this method like ascorbic acid and have a molar extinction coefficient in the same order as the latter.

† Numbers in parentheses = number of animals.

‡ Dye reduced with glutathione before injection.

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enzyme *in vivo* is of particular interest, since this compound does not have an enediol structure as do ascorbic acid and its analogues. Further evaluation of 2,6-dichlorophenolindophenol as an anti-scorbutic agent is now under investigation.

DISCUSSION

The tyrosyluria resulting from feeding tyrosine to vitamin C-deficient guinea pigs can now be explained in terms of an inhibition of *p*-hydroxyphenylpyruvic acid oxidase in the liver and kidney. Since it has been shown previously that *p*-hydroxyphenylpyruvic acid oxidase is inhibited *in vivo* by the injection of *p*-hydroxyphenylpyruvic acid (10), resulting in the accumulation and excretion of *p*-hydroxyphenylpyruvic acid, it appears likely that tyrosine also exerts its inhibitory effect via this α-keto acid. The ability of vitamin C and several related compounds to protect *p*-hydroxyphenylpyruvic acid oxidase from substrate inhibition, both *in vivo* and *in vitro*, is further evidence in accord with this mechanism.

It is of interest that guinea pigs need not be frankly scorbutic in order to exhibit inhibition of their *p*-hydroxyphenylpyruvic acid oxidase by tyrosine feeding. However, the susceptibility to inhibition increases as the vitamin C deficiency becomes more severe. The degree of enzyme inhibition depends upon two factors: the tissue concentration of vitamin C and the load of tyrosine given. These observations recall the findings of Painter and Zilva (20) in studies on the tyrosyluria of vitamin C-deficient guinea pigs. These authors found that the amount of *p*-hydroxyphenylpyruvic acid excreted was dependent upon the relative intake of tyrosine and of vitamin C.

The 4- to 5-fold increase in tyrosine transaminase activity induced by tyrosine feeding in the vitamin C-deficient animals would also favor the accumulation of *p*-hydroxyphenylpyruvic acid and help to perpetuate the metabolic defect. This increase in tyrosine transaminase might be an adaptive change secondary to the intake of substrate, but other possibilities must be considered, such as a protective (21) or activating effect (22, 23) of the amino acid. It should be noted that no increase in tyrosine transaminase activity occurred in the normal guinea pigs fed large amounts of tyrosine.

In view of these results, it is of interest to reconsider the origin of the tyrosyluria associated with feeding tyrosine or phenylalanine to premature infants. This tyrosyluria can be corrected by the administration of ascorbic acid (5), and it is reasonable to assume that inhibition of *p*-hydroxyphenylpyruvic acid oxidase by *p*-hydroxyphenylpyruvic acid accounts for the tyrosyluria found in these individuals.

Tyrosyluria has also been induced in guinea pigs by the administration of diethyldithiocarbamate (24). This agent, a potent inhibitor of *p*-hydroxyphenylpyruvic acid oxidase *in vitro* (7), also inhibits this enzyme *in vivo*, thus leading to the excretion of *p*-hydroxyphenylpyruvic acid in the urine when tyrosine or phenylalanine are fed. Inhibition of the oxidase by diethyldithiocarbamate takes place even in the presence of high tissue concentrations of ascorbic acid, and this compound appears to inhibit the oxidase directly.

The tyrosyluria reported in man in the unique case of tyrosinosis described by Medes (6) should also be mentioned. This condition has been assumed to be a metabolic disease in which the ability to further metabolize *p*-hydroxyphenylpyruvic acid is blocked, perhaps because of the lack of *p*-hydroxyphenylpyruvic acid oxidase. However, the exact nature of the metabolic defect has not been established (25).

The ability to block tyrosine metabolism at specific steps may be of value in further studies of the alternative pathways available for the metabolism of this amino acid. Induced metabolic blocks may also be useful in the treatment of metabolic disorders. For example, if a block could be induced at an earlier step than homogentisic acid oxidase, the pigmentation, ochronosis, and arthritis associated with alcaptonuria might be alleviated.

SUMMARY

1. The inhibition *in vivo* of *p*-hydroxyphenylpyruvic acid oxidase has been demonstrated in vitamin C-deficient guinea pigs, as well as severely scorbutic animals when fed extra tyrosine.

2. The excretion of *p*-hydroxyphenylpyruvic acid and other tyrosyl compounds in these animals can be attributed to the inhibition *in vivo* of *p*-hydroxyphenylpyruvic acid oxidase.

3. Inhibition of *p*-hydroxyphenylpyruvic acid oxidase in vitamin C-deficient animals can be prevented by the administration of ascorbic acid and by several other compounds such as 2,6-dichlorophenolindophenol, D-isoascorbic acid, and D-glucoascorbic acid. These compounds also protect the enzyme from inhibition by excess substrate *in vitro*.

4. The tyrosine transaminase activity is increased 4- to 5-fold in scorbutic animals fed tyrosine and this may help to maintain the metabolic defect.

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ACTIONS OF SODIUM ASCORBATE ON SMOOTH MUSCLE

BY

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Ascorbic acid is frequently added to food and drug preparations. There have, however, been very few studies on the pharmacological activity of the vitamin, although its anti-scorbutic properties have been studied in great detail.

Large doses of ascorbic acid inhibit the bronchospasm induced in guinea-pigs by spasmogens such as histamine, and exert a beneficial effect in pathological states such as anaphylaxis (Dawson & West, 1965a). Contractions of intestinal smooth muscle are also inhibited by large concentrations of the vitamin (Dawson, Hemsworth & Stockham, 1965). Further studies of the pharmacological effects of ascorbic acid on smooth muscle were therefore considered of interest.

METHODS

Bronchoconstriction in vivo

Guinea-pigs (300–500 g) were anaesthetized with chloralose (110 mg/kg intraperitoneally) and artificially ventilated by a constant volume pump through a tracheal cannula. Pressure in the trachea was recorded using a transducer system. Drugs were injected into a jugular vein.

Isolated guinea-pig ileum preparation

A segment of terminal guinea-pig ileum (2–2.5 cm) was suspended in aerated Tyrode solution at 32° C in a 10 ml. bath and contractions were recorded isotonically using a frontal writing lever with a magnification of 4 and a load of 1 g. Drugs were dissolved in 0.9% saline. Ascorbic acid was dissolved in distilled water and neutralized to pH 6–7 with sodium bicarbonate, unless otherwise stated in the text. The dose cycle for acetylcholine, 5-hydroxytryptamine and histamine was 3 min and the contact time 30 sec. The sodium ascorbate was mostly left in contact with the tissue for 5 min and then washed out before the next dose of spasmogen. Doses of all drugs are expressed in g/ml. bath volume.

Transmural stimulation of guinea-pig ileum

The method described by Paton (1955) was used.

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Diet deficient in ascorbic acid

Guinea-pigs were made deficient in ascorbic acid by feeding a diet of autoclaved Rank SG1 pellets, supplemented with vitamins A, B complex and D. Other animals received the same diet with ascorbic acid (50 mg/guinea-pig/day) included in the drinking water.

RESULTS

Effect of sodium ascorbate on bronchospasm in guinea-pigs

Intravenous histamine (5 μg/kg) produced a marked bronchoconstriction in guinea-pigs as indicated by an increase in tracheal pressure with constant volume lung inflations (Fig. 1). This bronchoconstriction was inhibited by sodium ascorbate (200 mg/kg) given 1 min previously. After 15 min, the response to histamine was potentiated and then, after a further 15 min, it had returned to control levels. Bradykinin (10 μg/kg), 5-hydroxytryptamine (5 μg/kg) and acetylcholine (5 μg/kg) also induced bronchoconstriction which was first inhibited and then potentiated by ascorbate. The potentiation and inhibition of bronchoconstriction induced by these spasmogens were not modified by depleting the tissues of catecholamines and 5-hydroxytryptamine with reserpine (5 mg/kg daily for 2 days), nor did β-blocking drugs, or acute adrenalectomy, have any effect (Dawson & West, 1965b).

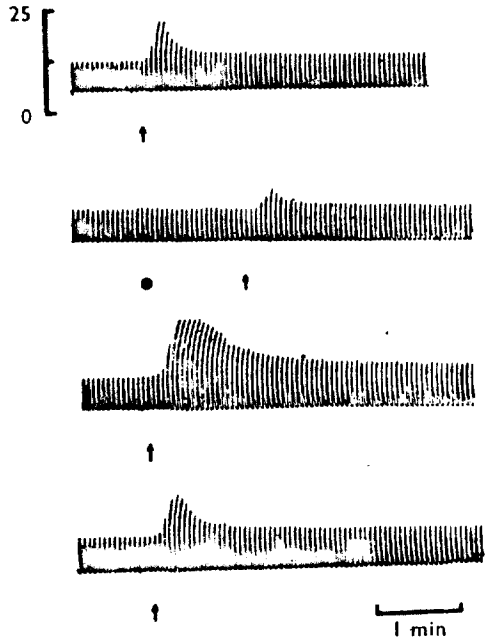

Fig. 1. This is a continuous record showing tracheal pressure in mm Hg in guinea-pigs. At the arrows 5 μg/kg histamine and at the dot sodium ascorbate (200 mg/kg) were injected intravenously. An increase in size of response represents bronchoconstriction. Note the initial inhibition of the histamine response followed by a marked potentiation.

Effect of sodium ascorbate on isolated guinea-pig smooth muscle

Contractions produced by acetylcholine (4×10^{-9}) increased three-fold after the addition of a single dose of sodium ascorbate (5×10^{-4}) (Fig. 2a). Increasing the dose of ascorbate to 10^{-3} resulted in an initial block which was followed by a two-fold increase in response to acetylcholine (Fig. 2b). Increasing the dose further to 5×10^{-3} produced a contraction *per se* but partially inhibited the effect of spasmogen for 15 min before a three-fold potentiation occurred (Fig. 2c). A single dose of 10^{-2} first produced a small contraction and then blocked the acetylcholine response, which failed to recover to the control level (Fig. 2d). Similar results were obtained using histamine and 5-hydroxytryptamine as spasmogens.

The degree of blockade of the effect of the spasmogens by ascorbate was a function of concentration and contact time of the vitamin—for example, 10^{-2} ascorbate for 5 min

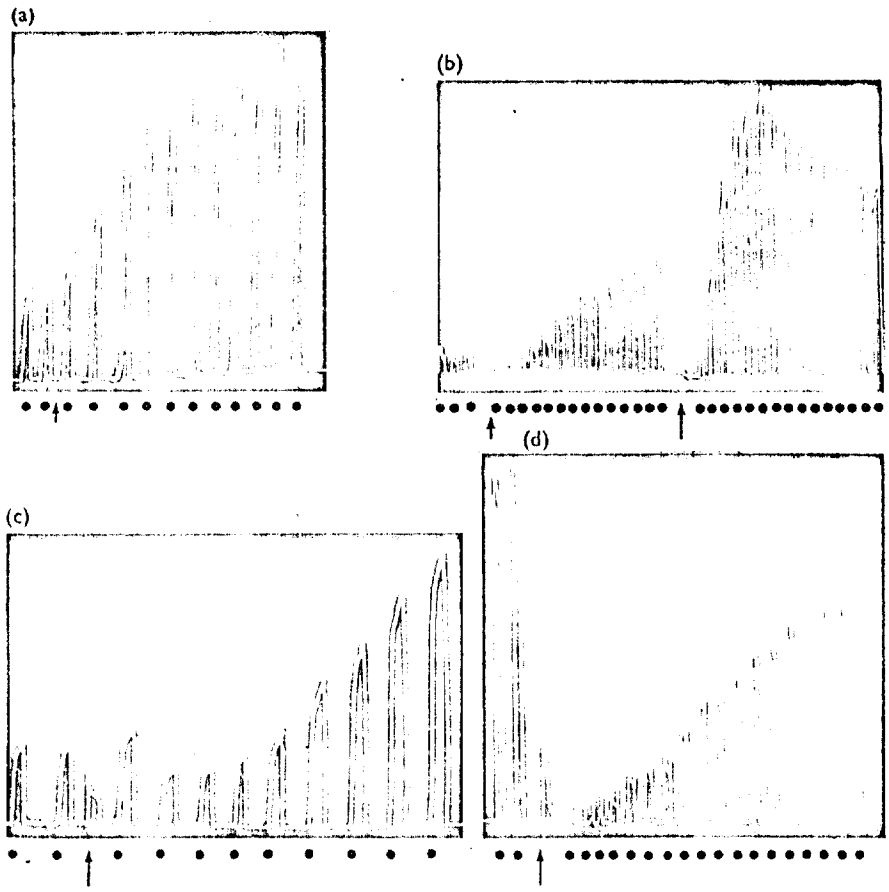

Fig. 2. Contractions of the guinea-pig ileum: at the dots acetylcholine 4×10^{-9}. (a) At the arrow 5×10^{-4} ascorbate. (b) At the arrows 10^{-3} ascorbate. (c) At the arrows 5×10^{-3} ascorbate. (d) At the arrow 10^{-2} ascorbate. Note the potentiation with the smaller doses and the inhibition with the larger doses of ascorbate.

gave a similar degree of inhibition to 10^{-3} ascorbate for 30 min. These values may be compared with 10^{-3} ascorbate for 5 min (Figs. 2b and d).

Sodium ascorbate produced contraction of guinea-pig ileum giving a straight line log dose/response curve, and tachyphylaxis was observed at a dose level of 1.6×10^{-2}, with subsequent recovery after a 15-min rest period (Fig. 3).

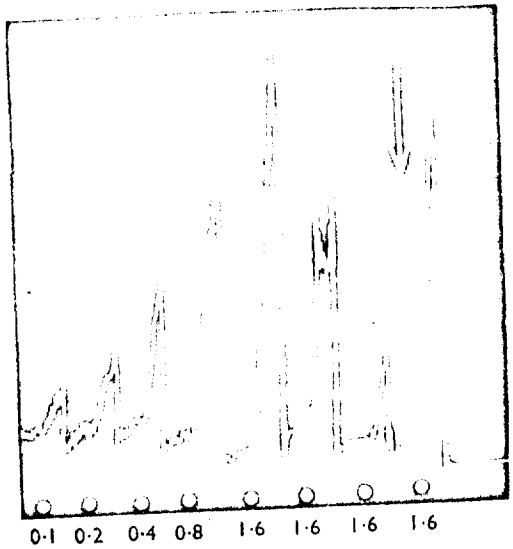

Fig. 3. Contractions of guinea-pig ileum with sodium ascorbate using a 3-min dose cycle: at the dots sodium ascorbate (doses $\times 10^{-2}$ g/ml.) was added to the bath and left in contact for 30 sec and then washed out. Note the tachyphylaxis to the concentration 1.6×10^{-2}. At the arrow the ascorbate was washed out and after a 15-min wait 1.6×10^{-2} ascorbate was again added to the bath.

This effect was probably not due to the antioxidant action of the vitamin, as sodium metabisulphite and thiourea had no spasmogenic effect themselves, nor did they affect the response of the tissue to spasmogens in low doses, although contractile responses were irreversibly blocked by large doses.

Ascorbate also produced potentiation (Fig. 4a) or blockade (Fig. 4b) or both blockade and potentiation (Fig. 4c) of the response to supramaximal stimulation of the transmurally stimulated guinea-pig ileum.

Effect of blocking drugs on the direct spasmogenic effect of sodium ascorbate on guinea-pig ileum

The spasmogenic action of sodium ascorbate was not modified by atropine (10^{-8}), mepyramine (5×10^{-7}) and 2-bromolysergic acid (10^{-7}) which respectively antagonized the responses to acetylcholine, histamine and 5-hydroxytryptamine. However, the sodium ascorbate response was blocked by papaverine (10^{-3}), a non-specific spasmolytic drug.

Effect of deficiency of ascorbic acid on the sensitivity of guinea-pig ileum to spasmogens

The log dose/response curves obtained with acetylcholine on segments of ileum from 21-day ascorbic acid deficient and control guinea-pigs fed the complete diet are shown in Fig. 5. This ten-fold difference was also seen when comparisons of other segments of ileum were made—for example, the duodenal region or the midileal region—of deficient and control animals. The sensitivity of the deficient tissue could be restored either by adding sodium ascorbate to the isolated tissue (5×10^{-4}) or by adding ascorbic acid to the diet (50 mg/guinea-pig/day). Similar results were obtained with other spasmogens.

DISCUSSION

Although the doses of ascorbate used in these experiments are large, children and adults ingesting certain foods and vitamin preparations may have an intake approaching 200 mg/kg, which was the dose used in the guinea-pig bronchospasm experiment. It is also important to note that to maintain sensitivity of the ileum to spasmogens, ascorbic acid was needed in the diet, and in fact, by ensuring that the diet contained enough ascorbic acid for maintenance of health of the guinea-pig, sensitivity of intestinal muscle to spasmogenic drugs was maintained. The intestinal muscle from ascorbic acid-deficient guinea-pigs shows a marked decrease in sensitivity to spasmogens, well before clinical signs of scurvy are induced. In addition, sodium ascorbate exerts several effects on smooth muscle which are dose dependent: it may inhibit or potentiate the action of spasmogens, or have a direct stimulant action *per se*.

Ascorbic acid has been used clinically for many years in the treatment of various conditions, including many concerned with muscular disorders. Hirata & Suzuki (1935, 1937) described a deficiency of ascorbic acid in the cerebrospinal fluid of patients suffering from progressive muscular atrophy; the administration of ascorbic acid improved this condition, increasing the glycogen content of muscle. Piéry, Cordier & Enselme (1940) showed that deposition of liver glycogen was also augmented. Spitzer (1947) found that small doses of ascorbic acid were useful in the first stage of labour as an oxytocic agent, and often potentiated a small dose of pitocin, whereas larger doses of ascorbic acid had little effect. Our findings—that larger doses of ascorbate are inhibitory—may explain this observation.

Many workers have used ascorbic acid as an antihistamine in the treatment of allergic reactions, with varying degrees of success. Rosa & Parenti (1950) used the vitamin in bronchial asthma and urticaria with some success, but Naranjo (1952) and Herxheimer (1955) obtained no protection against anaphylaxis in experimental animals. Hochwald (1935), van Niekerk (1937), Aron (1948) and Dawson & West (1965a) all showed a short-term protective action of ascorbic acid against anaphylaxis in guinea-pigs. These protective effects have usually been obtained in guinea-pig and man in allergic conditions where contraction of the bronchial smooth muscle is of prime importance and it is suggested that the protection is due to a direct action of the vitamin on the smooth muscle.

Ascorbic acid may have a synergistic effect when administered with antihistamines against allergic phenomena (Dawson & West, 1965a) since rat anaphylaxis is completely inhibited by pretreatment with a mixture of these drugs. This result may be correlated with the blocking action of ascorbate on guinea-pig smooth muscle preparations against the agonist histamine as well as against other agonists such as acetylcholine and

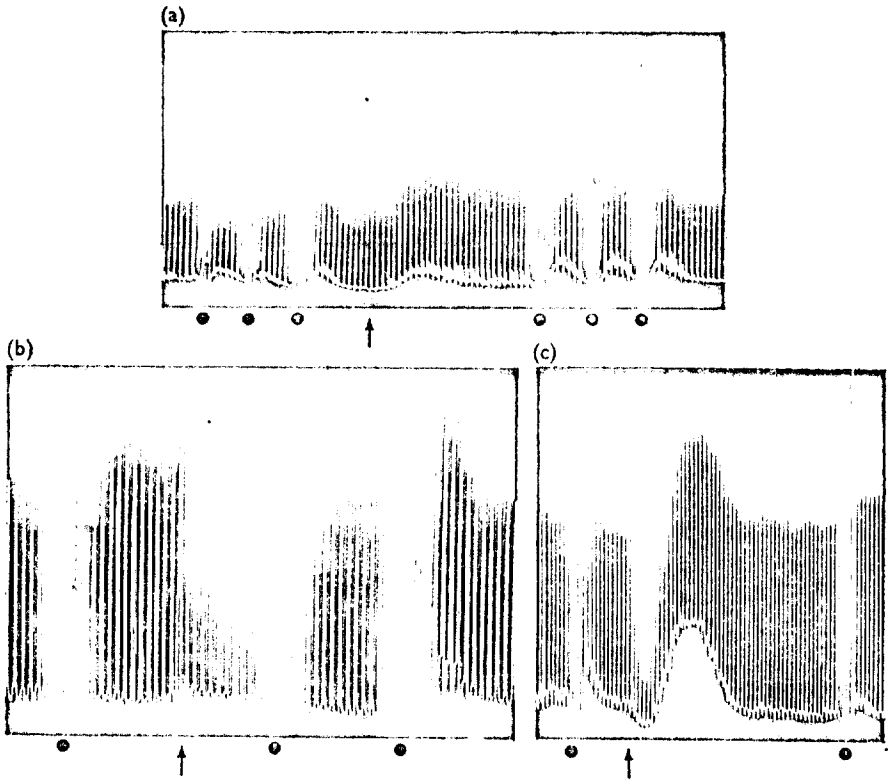

Fig. 4. Contractions of guinea-pig ileum elicited by transmural stimulation. (a) At the dots 10^{-8}, 2×10^{-8}, 4×10^{-8}, acetylcholine respectively and 10^{-3} ascorbate at the arrow. (b) At the dots 8×10^{-9} acetylcholine and 10^{-2} ascorbate at the arrow. (c) At the dots 8×10^{-3} acetylcholine and 5×10^{-3} ascorbate at the arrow. Note the potentiation with the smaller doses and the inhibition with the larger doses of ascorbate.

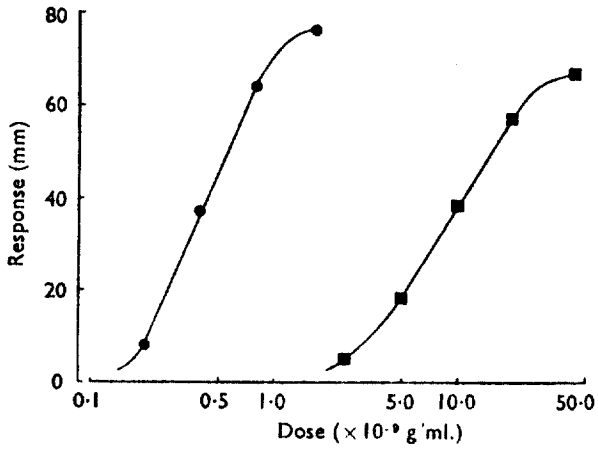

Fig. 5. Log dose/response curves for acetylcholine using terminal guinea-pig ileum preparations from guinea-pigs fed the complete diet (○——●) and from guinea-pigs maintained 21 days on the ascorbic acid deficient diet (□——■).

5-hydroxytryptamine. Preliminary clinical studies show that ascorbic acid itself can partially relieve the spasm of bronchial smooth muscle in both hay-fever and bronchial asthma.

SUMMARY

1. Sodium ascorbate has a direct stimulant action on smooth muscle.

2. Spasmogenic responses are potentiated by sodium ascorbate in low concentrations and are inhibited by high concentrations of this substance.

3. The actions of sodium ascorbate are not modified by atropine, mepyramine, 2-bromolysergic acid or reserpine.

We are grateful to Dr. G. B. West for his helpful criticism in the preparation of this manuscript.

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Ascorb. # 149

Chemotherapy - Stereochemical configuration of the organic support and anti-tumoral activity of metallo-ascorbic complesex. Note by Frenan Arloing, Albert Morel, Andre Jossebrand and Louis Perrot.

The soluble complex salts, whose anti-tumoral activity with respect to experimental and human cancers we have reported (1), were prepared from l-ascorbic acid, to the study of which we were led by the logical development of our work on the activity of the constituents of the adrenals. We wished to interpret the chemical mechanism of this action and attempt to attach it to certain groups of constituent atoms of these complicated molecules.

We know (2) that the anti-scorbutic activity appears only in the bodies showing, in addition to an enediol grouping that may constitute a reversible Redox system, conjugated with a lactonic grouping, a special stereochemical configuration. This should contain a certain assymmetric carbon, the fourth, which should be situated in the straight configuration, while the structure in space of another carbon, likewise assymmetrical, the fifth, also has a certain amount of importance, but to a lesser extent. Does the antineoplasic action of the metallo-ascorbic complexes, depend on the same stereochemical conditions, or is it to be attached only to the existence of the Redox system, reinforced by the intimate presence of metals capable of passing from one state of oxidation to another? We sought the answer in a comparison of the anti-tumoral activity (the essential of which is the disinfiltrating power of the noeplasms) carried on by the complexes derived from dehydrascorbic acid, the first product of oxidation of vitamin C, with the activity of the soluble complex salts which we prepared from the first product of oxidation of iso-vitamin C or d-arabo-ascorbic acid. The configuration of the latter, according to Haworth and

Hirst (3) and also according to Reichstein (loc. cit.) differ from that of l-ascorbic acid only by a right-hand, in lieu of a left-hand position of the hydroxyl fixed on the fifth carbon. This isovitamin, prepared synthetically from 2-keto gluconic acid (4), has, according to Reichstein as well as Dalmer and Moll (loc. cit.) an antiscorbutic power twenty times less than vitamin C.

We have noted that the ferric complexes derived therefrom, have with respect to tumors, an effect which is substantially the same as that of the derivatives of l-ascorbic acid. The quantitative differences of their anti-neoplasic effects (less rapidity at the start, but longer-lasting action and less risk of abrupt relapse after cessation of the treatment) are far from being on the order of size of those observed in connection with the anti-scorbutic power.

The influence of the structure of the fifth carbon atom is therefore small. The next thing to do is elucidate that of the fourth carbon.

We therefore prepared soluble complex salts from another 2-3-ene diol, oxy-tetronic acid in which, this atom, not being assymmetric, can exert no effect.

We are continuing our study of the action of the latter complexes with respect to neoplasms, and we will present these results at a subsequent date.

But our first observations, detecting a manifest disinfiltration activity in these substances, permit us to make it known, now, that this activity does not seem to depend on the structure of the fourth carbon atom, and that it thereby differs clearly from the anti-scorbutic power, which oxy-tetronic acid completely lacks.

The role played by the metals associated with the Redox system in the anti-tumoral activity of the complexes which we are studying, therefore becomes preponderant in the disinfiltration power.

But the fact remains that the eutrophic action seems to belong quite

particularly to the derivatives of l-ascorbic acid. This is the case in particular of the dehydrascorbic ferro-magnesian complex, whose happy effect we have noted on the general state of cancerous subjects, as well as the favorable influence on scleroses in plaque (5).

The session adjourned at 4:20 pm.

E.P.

Footnotes

(1) Arloing, Morel and Josserand, Comptes rendus, 201, 1935, pp 436 and 745; 202, 1936, page 598.

(2) See lecture by P. Karrer, Bull. Soc. chim., 5th series, 2, 1935, page 394 and Actual. chim. et industr., 34, 1935, page 1030; see also Reichstein, Helvet., 17, 1934, page 512, and Dalmer & Moll, Z. physiol. Chem., 222, 1933, p.117.

(3) J. Roy. Soc., London, 1934, p. 62.

(4) Ber. d. chem. Ges., 66, 1933, page 1054; 67, 1934, pages 555 and 1239.

(5) J. Lepine, F.Arloing, A. Morel and Josserand, Bull. Acad Medicine, 116, 1936, page 274.

(Translated by Carl Demrick Associates,Inc/GCT/t)

Ascorb. #149

variante du bacille d'Aertrycke qui renferme un antigène O particulier : identique à l'antigène O de l'Aertrycke typique, quant à sa spécificité, il en diffère par sa plus grande richesse en acides gras, par sa moindre teneur en polysaccharide responsable de la spécificité et par son instabilité en solutions aqueuses. Mais lorsqu'une telle variation se produit dans l'antigène O, elle ne paraît dépendre, en aucune façon, d'un changement simultané quelconque dans l'antigène H.

CHIMIOTHÉRAPIE. — *Configuration stéréochimique du support organique et activité antitumorale des complexes métallo-ascorbiques.* Note de MM. **Fernand Arloing, Albert Morel, André Josserand** et **Louis Perrot.**

Les sels complexes solubles, dont nous avons signalé (¹) l'action antitumorale vis-à-vis des cancers expérimentaux et humains, étaient préparés à partir de l'*acide l-ascorbique*, à l'étude duquel nous avons été conduits par le développement logique de nos travaux sur l'activité des constituants des surrénales. Nous avons voulu interpréter le mécanisme chimique de cette action et essayer de le rattacher à certains groupements d'atomes constitutifs de ces molécules compliquées.

On sait (²) que le pouvoir antiscorbutique n'apparaît que chez les corps qui présentent en plus d'un groupement énediol susceptible de constituer un système Rédox reversible, conjugué avec un groupement lactonique, une configuration stéréochimique spéciale. Celle-ci doit comporter un certain carbone asymétrique, le quatrième, lequel doit se trouver dans la configuration droite, tandis que la structure dans l'espace d'un autre carbone également asymétrique, le cinquième, a encore une certaine importance, mais beaucoup moindre. L'action antinéoplasique des complexes métallo-ascorbiques dépend-elle des mêmes conditions stéréochimiques ou n'est-elle à rattacher qu'à l'existence du système Rédox, renforcé par la présence intime de métaux susceptibles de passer d'un état d'oxydation à

(¹) Arloing, Morel et Josserand, *Comptes rendus*, **201**, 1935, p. 456 et 745; **202**, 1936, p. 598.

(²) Voir conférence de P. Karrer, *Bull. Soc. chim.*, 5e série, **2**, 1935, p. 934, et *Actual. chim. et industr.*, **34**, 1935, p. 1030; voir aussi Reichstein, *Helvet.*, **17**, 1934, p. 513, et Dalmer et Moll, *Zschr. physiol. Chem.*, **222**, 1933, p. 117.

un autre? Nous avons demandé la réponse à la comparaison de l'activité antitumorale (dont l'essentiel est le pouvoir désinfiltrant des néoplasmes) exercée par les complexes dérivés de l'acide déhydrascorbique, premier produit d'oxydation de la vitamine C, avec l'activité des sels complexes solubles que nous avons préparés à partir du premier produit d'oxydation de l'isovitamine C ou *acide d-arabo-ascorbique*. La configuration de celui-ci, d'après Haworth et Hirst [1] et aussi d'après Reichstein (*loc. cit.*), ne diffère de celle de l'acide *l*-ascorbique que par la position droite au lieu de gauche de l'hydroxyle fixé sur le cinquième carbone. Cette isovitamine, préparée synthétiquement à partir de l'acide 2-cétogluconique [2], présente, d'après Reichstein comme d'après Dalmer et Moll (*loc. cit.*), un pouvoir antiscorbutique vingt fois moindre que celui de la vitamine C.

Nous avons constaté que les complexes ferriques qui en dérivent possèdent vis-à-vis des tumeurs une action sensiblement la même que celle des dérivés de l'acide *l*-ascorbique. Les différences quantitatives de leurs effets antinéoplasiques (moindre rapidité du début, mais action plus soutenue et risque moindre de récidive brusque après cessation du traitement) sont loin d'être de l'ordre de grandeur de celles observées en ce qui concerne le pouvoir antiscorbutique.

L'influence de la structure du cinquième atome de carbone est donc faible. Reste à élucider celle du quatrième carbone.

Nous avons alors préparé des sels complexes solubles à partir d'un autre 2-3-énediol, l'*acide oxy-tétronique* chez qui cet atome, n'étant pas asymétrique, ne peut exercer d'influence.

Nous poursuivons l'étude de l'action de ces derniers complexes vis-à-vis des néoplasmes, et nous communiquerons prochainement ces résultats.

Mais nos premières constatations, décelant chez ces corps une activité désinfiltrante manifeste, nous autorisent à faire connaître dès aujourd'hui que cette activité ne semble pas dépendre de la structure du quatrième atome de carbone et qu'elle se différencie nettement par là du pouvoir antiscorbutique dont l'acide oxy-tétronique est totalement dépourvu.

Le rôle joué par les métaux associés au système Rédox dans l'activité antitumorale des complexes que nous étudions devient donc prépondérant dans le processus de désinfiltration.

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(1) *Journ. roy. Soc. London*, 1934, p. 62.

(2) *Ber. d. chem. Ges.*, 66, 1933, p. 1054; 67, 1934, p. 555 et 1239.

Il reste cependant que l'action eutrophique semble appartenir tout spécialement aux dérivés de l'acide *l*-ascorbique. C'est en particulier le cas du complexe ferrico-magnésien déhydrascorbique dont nous avons noté l'heureux effet sur l'état général des cancéreux et l'influence favorable sur les scléroses en plaque (¹).

La séance est levée à 16h20m.

E. P.

(¹) J. Lépine, F. Arloing, A. Morel et Josserand, *Bull. Acad. Médecine*, 116, 1936, p. 374.

ILLUSTRATIVE PROTOCOL—(*Concluded*)

TIME	DIAMETER OF (RIGHT) PUPIL IN C.D. IRIS	BLOOD PRESSURE (SYSTOLIC)	PROCEDURES AND REMARKS
	mm.	*mm. Hg*	
3/04/44	5.5	180	
3/04/49	5.5	190	
3/05/09	5.5	200	
3/06/45	5.5	180	Pulse rate rapid
3/07/55	5.5	150	
3/09/20	5.5	100	
3/11/17	5.5	90	
(*8*)			After atropine primary vaso-depression no longer present
3/22/27			Atropine 1 mgm. intramuscularly
(*9*)			
3/24/00	5.5	110	Methyl doryl 0.3 mgm. intravenously
3/24/08	5.5	110	Contraction in left face
3/24/12	5.5	Slight rise	
3/24/50	5.5	120	
3/25/05	5.5	110	There was no change in pupils
(*10*)			Large doses of methyl doryl produced delayed mydriasis
3/43/15	5.5	110	Dimethyl doryl 0.8 mgm. intravenously
3/43/21	5.5		Marked contraction in denervated face
3/43/24	5.5	Abrupt rise	
3/43/32	5.5	180	
3/43/40	5.5	260	
3/43/44	5.8	280	Pulse accelerated. Marked pallor of mucous membrane
3/43/55	Dilating		
3/44/06	7.5	Declining	
3/44/20	7.8		
3/44/30	7.0	220	
3/45/00		180	
3/45/55	6.5	150	
3/47/28	6.2	115	
3/47/43	6.0	110	
3/48/23	6.0	100	
3/51/12	5.5	90	
(*11*)			
3/53/29			Dimethyl doryl 0.2 mg. per kilo again produced an increase in B.P. to 240 mm. but no change in pupil. Doses above 0.3 mgm. per kilo were lethal

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(1) HUNT, R., AND R. R. RENSHAW, THIS JOURNAL, **35**: 99, 1929; **37**: 177, 1929; **37**: 193, 1929; **44**: 63, 1932; **44**: 151, 1932; **48**: 105, 1933; **51**: 237, 1934; **58**: 140, 1936.
(2) SPRINSON, D. B., J. Am. Chem. Soc., **63**: 2249, 1941.
(3) BENDER, M. B., AND E. A. WEINSTEIN, Am. J. Physiol., **130**: 268, 1940.
(4) CANNON, W. B., AND A. ROSENBLUETH, Autonomic Neuro-Effector System. Macmillan, N. Y., 1936.
(5) GLICK, D., J. Biol. Chem., **130**: 527, 1939.

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PHARMACOLOGY AND CHEMISTRY OF SUBSTANCES WITH CARDIAC ACTIVITY[1]

II. EFFECT OF *l*-ASCORBIC ACID AND SOME RELATED COMPOUNDS AND OF HYDROGEN PEROXIDE ON THE ISOLATED HEART OF THE FROG

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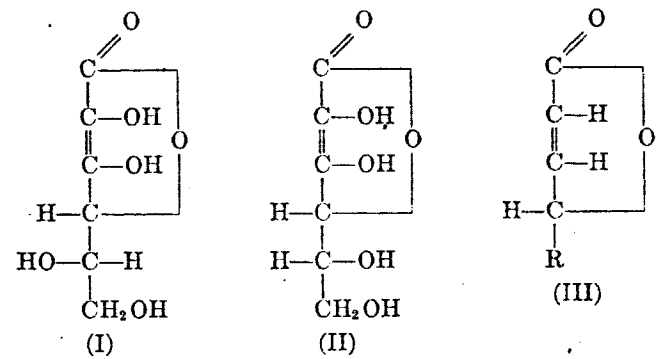
l-Ascorbic acid (I) and *d*-iso-ascorbic acid (II) can be considered as $\alpha\ \beta$-unsaturated lactones related in structure to the $\alpha\ \beta$-unsaturated lactones of type III. It therefore seemed possible that their action upon the isolated frog heart



might be similar to the action of the angelicalactones which we recently described (1). Urban and Peugnet (2) studied the action of *l*-ascorbic acid on the frog heart employing the technique of Straub and observed a "beat strengthening" action, an acceleration in heart rate, and, although not consistently, an increase in the "diastolic tonus." Peugnet in a further study (3) found that the presence of small amounts of copper was necessary and that the product formed when *l*-ascorbic acid was oxidized with iodine was ineffective. Preliminary experiments with our modification of Straub's technique confirmed some of the observations of Urban and Peugnet and showed that an adequate concentration of *l*-ascorbic acid administered continuously caused irreversible systolic standstill, as was observed with the angelicalactones (4). The present paper deals with the results of a more detailed study of this action and with the analysis of its cause.

METHOD. The experiments were carried out on frogs (mostly male) of the species *Rana pipiens* between February and May and October and November, 1942. A few preliminary experiments were conducted with the Clark solution used in our previous experiments. For

[1] This work was carried out under the auspices of the University Committee on Pharmacotherapy.

the experiments described here, however, a well-buffered solution was needed. We chose a bicarbonate buffer solution similar to that of McLean and Hastings (5), of the following composition: sodium chloride 0.5%; potassium chloride, 0.014%; calcium chloride anhydrous, 0.011%; and sodium bicarbonate, 0.23%. This solution was saturated with a gas mixture containing either 95% oxygen and 5% carbon dioxide or 95% nitrogen and 5% carbon dioxide. In order to prevent a change in the oxygen and carbon dioxide tension, rubber bags filled with the gas mixture being used were attached to the Mariotte bottles which served as reservoirs. The heart cannula had to be adapted to our special needs (see fig. 1). The fluid dropped into the wide part of the cannula at A, and, with the heart attached to the cannula, left it by way of the side tube at C. The capacity of the cannula from the level of the fluid in A to the side tube C was approximately 2.5 cc. The side tube C was so arranged that the height of the fluid column above the tip of the cannula was not more than 3 to 4 cm. The hydrostatic pressure to which the ventricle was exposed was therefore higher than 2 to 3 cm. as in Straub's original arrangement (6) but lower than in our own previous technique (1). A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the

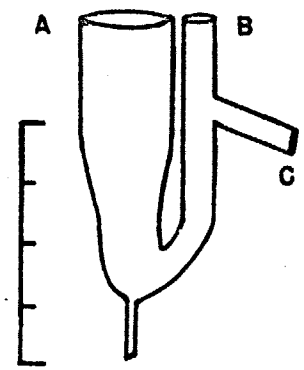


FIG. 1. CANNULA FOR ISOLATED FROG HEART (SCALE IN CM.)
In order to remove fluid from close proximity to the heart a hollow needle can be inserted through the opening at B. For further description see text.

fluid in the wide part of the cannula in order to maintain the carbon dioxide tension. Moreover, this procedure guaranteed the necessary supply of oxygen to the heart in those experiments in which the solution in the Mariotte bottle was kept under nitrogen. The rate of replacement of the solution in the cannula was 2 to 2.5 cc. per minute.

The *l*-ascorbic acid and the related compounds were dissolved in distilled water and neutralized with sodium bicarbonate, the sodium salts being used in making up the solutions. As some of the substances tested are readily destroyed while standing in a solution containing oxygen, concentration, unless otherwise specified, refers to the initial concentration of the tested agent. Repeated tests with the glass electrode indicated that the pH range of of the fluid entering the heart was between 7.5 and 7.8 in all experiments.

The *l*-ascorbic acid and the *d*-iso-ascorbic acid used were the crystalline synthetic products.[2] The dimethylascorbic acid was prepared by the action of excess diazomethane on ascorbic acid (7); the product was not obtained in crystalline form, but iodine titration showed that only 0.2% reducing material was present.

[2] We are indebted to Merck & Co., Rahway, N. J., and to Hoffman-La Roche Inc., Nutley, N. J., for a supply of *l*-ascorbic acid, and to Eastman Kodak Company, Rochester, N. Y., for a supply of *l*-ascorbic acid and of *d*-iso-ascorbic acid.

The dehydroascorbic acid was first prepared by titration of ascorbic acid with iodine solution and later by the action of quinone on ascorbic acid (8). Both methods yielded material unsuited to our experiments for reasons which will be discussed later. Satisfactory results were obtained by oxidation of *l*-ascorbic acid (or *d*-iso-ascorbic acid) with Norit in the presence of acetic acid. One hundred milligrams of ascorbic acid was dissolved in 30 cc. of 0.6% acetic acid solution, Norit was added and oxygen was bubbled through the mixture. After 1½ hours the ascorbic acid concentration, judging from the reducing power of the solution, had decreased from 330 mgm. % to less than 1 mgm. %. After removing the Norit by filtration sodium bicarbonate was gradually added to the solution until pH 6 was reached. This approximately isotonic sodium acetate solution containing the dehydroascorbic acid was used for the experiments.

The *l*-ascorbic acid and *d*-iso-ascorbic acid were estimated in some experiments by iodimetry, in others colorimetrically with 2,6-dichlorophenol indophenol, according to Mindlin and Butler (9), using a Klett-Summerson photoelectric colorimeter.

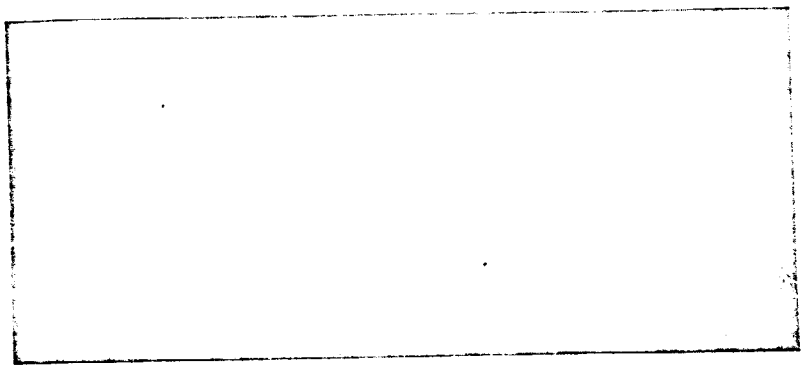


FIG. 2. FROG HEART (CANNULA OF FIG. 1)
Bicarbonate buffer solution saturated with 95% oxygen and 5% carbon dioxide. Temperature, 24–25°C. At sign, replacement with *l*-ascorbic acid 1:10,000 begun. The figures above the time line represent heart rate per minute. The figures below, which go with the heavy marks, indicate in minutes the periods of arrest of the drum. Time is recorded in 10-second intervals.

RESULTS. I. *Effect of l-ascorbic acid.* Altogether 36 experiments were carried out, 24 of these with a concentration of 1:10,000. The characteristic sequence of changes is shown in figure 2.

When *l*-ascorbic acid in an initial concentration of 1:2,000 to 1:100,000 was perfused through the isolated frog heart, the first characteristic change was an increase in the height of contraction. This as a rule occurred without a consistent change in rate. After some time the systolic height of contraction began to decrease, and shortly afterwards, or sometimes simultaneously, the diastolic relaxation became incomplete. The amplitude of contraction was usually further reduced at this time by an increase in rate. In some hearts the acceleration was slight, but in others increases up to 250% of the initial rate were observed. Intensity of systolic effect and increase in heart rate did not run parallel. Finally the ventricle stopped in systole while the atria and the sinus continued beating.

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Contrary to our experience with the angelicalactones, it was not possible to establish a clear relation between concentration of ascorbic acid and time taken to cause systolic standstill. It was also noted that dissolving *l*-ascorbic acid in Clark solution made the substance less potent than dissolving it in the bicarbonate buffer solution saturated with the oxygen-carbon dioxide mixture.

The preliminary period of observation during which the heart was supplied with the normal bicarbonate buffer solution usually extended from 10 to 30 minutes. When the continuous replacement of the normal solution was carried on for longer periods up to several hours, the systolic effect and the increase in heart rate still appeared, while a prolonged period of washing decreased or abolished the ability of the heart to respond to the *l*-ascorbic acid solution with an increase in the height of contraction.

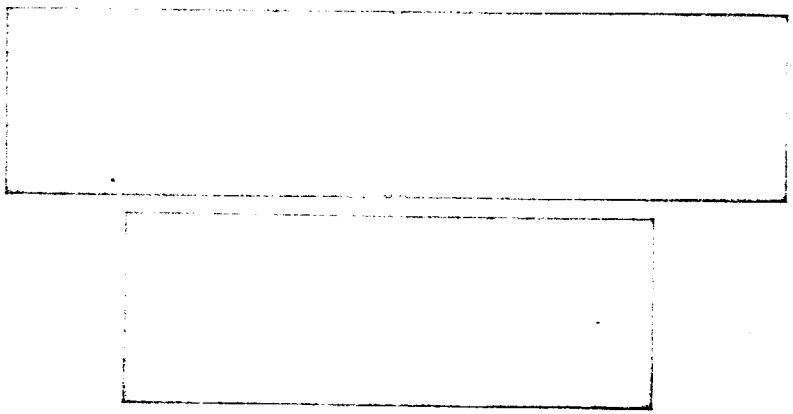


FIG. 3. ISOLATED FROG HEARTS (CANNULA OF FIG. 1)

Bicarbonate buffer solution saturated with 95% oxygen and 5% carbon dioxide. Same *l*-ascorbic acid solution 1:10,000 was used for replacement in hearts A, B, and C. The solution was prepared at 9:55 a.m. At *A*, replacement started 10:23 a.m.; at *B*, replacement started at 12:45 p.m.; at *C*, replacement started at 3:40 p.m. Figures above the tracings represent concentration of *l*-ascorbic acid in mM/liter. Figures below the tracings represent heart rate per minute. Time in half-hour intervals. Room temperature, 24.2–26.7°C.

In a number of experiments a solution with an initial concentration of *l*-ascorbic acid 1:10,000 was run through several hearts successively to obtain information concerning the speed of destruction of *l*-ascorbic acid and the relation between actual *l*-ascorbic acid concentration and intensity of effect. This procedure is illustrated in figure 3. Initially the solution used in this experiment contained 0.55 millimols of *l*-ascorbic acid per liter; when perfusion of heart A was started the concentration was 0.36 millimols per liter. It had reached 0.05 millimols per liter at the time of systolic standstill of heart A. This solution still caused all the characteristic effects in heart B, while in heart C, when the concentration had dropped to 0.02 millimols per liter, or less than 1:200,000, only the initial increase in the amplitude of contraction was observed. While in hearts A and B the heart rate increased, no significant change in rate occurred in heart C.

When the oxidative destruction of *l*-ascorbic acid was prevented by saturating the solution in the Mariotte bottle with the nitrogen-carbon dioxide mixture (bubbling oxygen-carbon dioxide mixture only through the solution in the cannula), the action upon the heart was greatly diminished or entirely abolished. In such experiments the *l*-ascorbic acid concentration, which initially was between 0.5 and 0.55 millimols per liter, decreased not more than 20% within 6 hours, or between 3 and 4% per hour. While the height of contraction diminished gradually, the heart still dilated fully in diastole. When the nitrogen in the Mariotte bottle was replaced by oxygen and destruction proceeded at a high rate, the systolic effect became noticeable after the characteristic latency

TABLE 1

Exp. March 11, 1942. Solution saturated at start with 95 per cent nitrogen and 5 per cent carbon dioxide

TIME	*l*-ASCORBIC ACID CONCENTRATION		HEART RATE	REMARKS
	From bottle	From heart		
min.	*mM/liter*	*mM/liter*		
0	0.5		60	
2				Replacement started
16		0.47	53	Height of excursion diminished
42		0.46	50	Height of excursion diminished
45	0.48			
64	0.49		50	Height of excursion further diminished
66		0.45	50	
70	Oxygenation started in Mariotte bottle with oxygen-carbon dioxide mixture			
73–90			48	Slight increase in height of excursion
109	0.34		58	Height of excursion diminished
110		0.33		
139	0.22		60	
141		0.23		
165			50	Systolic effect begins
181	0.14		45A 14V	Systolic effect marked (A = atria; V = ventricle)
185		0.16	54A	Ventricle stops in systole

period; the heart rate increased, and systolic standstill eventually terminated the activity of the heart. Table 1 shows the results of such an experiment. In this case *l*-ascorbic acid content was estimated in the Mariotte bottle as well as in the fluid from the cannula after it had passed the heart. With a replacement rate of 2.1 to 2.5 cc. as in this experiment, the difference in concentration was within the error of the method of estimation, and at this rate of replacement the concentration in the reservoir corresponded closely to the concentration entering and leaving the heart.

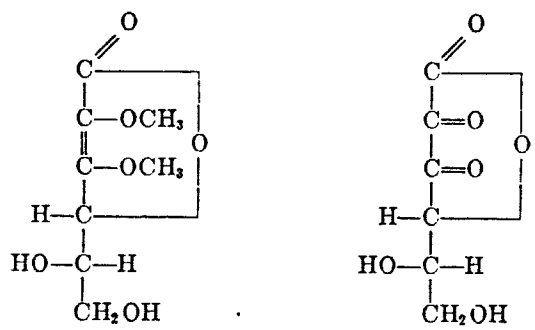
It could be argued that the effect upon the heart might not appear because of a decrease in the oxygen tension of the perfusion fluid, and consequently in the heart itself, although our technique makes this unlikely. That this argument is

not valid was proved in the following way: A solution of *l*-ascorbic acid 300 milligram % (or about 1:330) was made with bicarbonate buffer solution saturated with 95% nitrogen and 5% carbon dioxide, of which it was found that the reducing power did not change within three hours. This solution was infused continuously through a capillary tubing at a rate of about 0.1 cc. per minute into the wide part of the cannula (at A) through which flowed, at a rate of 2 to 2.5 cc. per minute, bicarbonate buffer solution saturated with 95% oxygen and 5% carbon dioxide. The continuous infusion of the small amount of the concentrated *l*-ascorbic acid solution—constituting about 4% of the total solution—was directed into the vigorous stream of oxygen-carbon dioxide bubbles passing through the fluid in the cannula to ensure uniform distribution. The fluid collected from the side tube (at C) in four such experiments, lasting for 2½ to 3 hours, maintained concentrations of *l*-ascorbic acid ranging from 1:7,000 to 1:9,000 for the duration of the experiments. The only significant change in the activity of the heart observed was a decrease in rate and a diminution of the height of contraction, such as commonly occurred when the experiments were conducted for the same period of time using the bicarbonate buffer solution alone.

II. *Action of d-iso-ascorbic acid.* *d*-Iso-ascorbic acid, or *d*-arabo-ascorbic acid, (II) differs biologically from *l*-ascorbic acid (I), the former having only about one-twentieth of the antiscorbutic activity of the latter (10). While the unsaturated lactone ring is of the same configuration as in (I) the position of the hydroxyl group on C_5 is changed.

In 15 experiments it was found that *d*-iso-ascorbic acid had qualitatively and quantitatively an effect on the frog heart similar to that of *l*-ascorbic acid. The action was diminished or prevented when the oxidative destruction was interfered with by saturating the solution of *d*-iso-ascorbic acid with nitrogen-carbon dioxide mixture.

III. *Action of dimethylascorbic acid and dehydroascorbic acid.* In order to find out whether chemical changes within the lactone ring might be involved in making *l*-ascorbic acid (or *d*-iso-ascorbic acid) active upon the frog heart, dimethylascorbic acid (IV) and dehydroascorbic acid (V) were tested.



(IV) (V)

Two experiments were made with dimethylascorbic acid in bicarbonate buffer solution saturated with oxygen-carbon dioxide mixture, one with a concentration 1:5,000 (replaced for 4 hours) and one with a concentration 1:3,000 (replaced for 7 hours). No significant effect could be observed on amplitude or rate; and no systolic effect was noticeable at the end of the experiments.

Dehydroascorbic acid prepared by titration of ascorbic acid with iodine solution in acetate buffer was unsuitable for our experiments. Preliminary tests showed that the iodine solution was not toxic to the heart when neutralized and added to the bicarbonate buffer solution in such an amount as would be required to make a solution of dehydroascorbic acid 1:10,000. But the solution of the end-products from *l*-ascorbic acid oxidation with this procedure impaired the activity of the heart, leading to a decrease in the amplitude of contraction and to a marked, although transient, irregularity of the heart rate.

The solutions resulting from the oxidation of *l*-ascorbic acid with quinone proved not toxic; however, they produced results on the frog heart qualitatively similar to those obtained with *l*-ascorbic acid itself under conditions which excluded the presence of *l*-ascorbic acid as well as of dehydroascorbic acid. As these solutions still contained 2 to 2.5% reducing material, it was necessary to ascertain whether or not their slight activity could be attributed to contamination with hydroquinone, particularly as Richter (11) had already shown that quinone and other oxidizing agents exerted a systolic effect upon the isolated frog heart. It was found in six experiments that the action of hydroquinone was similar to that of *l*-ascorbic acid and that concentrations as low as 1:600,000 to 1:1 million caused an initial increase in the height of contraction and that systolic standstill terminated the heart activity within 3 to 6 hours. The effect of hydroquinone, however, was not materially influenced by saturating the solution in the Mariotte bottle with nitrogen-carbon dioxide mixture instead of with oxygen-carbon dioxide mixture.

The preparation of dehydroascorbic acid with Norit in the presence of acetic acid, described above under *methods*, yielded a slightly acid solution devoid of poisonous effects which we believe was sufficiently stable for our purposes. As the halflife of dehydroascorbic acid at 38°C. and pH 7.2 is only two minutes (12) and is probably not very much longer at room temperature (22 to 25°C.) and pH 7.5 to 7.8, it was necessary to apply dehydroascorbic acid to the heart so that it was exposed to the alkaline medium of the bicarbonate buffer solution for only a very short time. This was achieved in the following way: The approximately isotonic sodium acetate solution of pH 6 containing 1:300 dehydroascorbic acid was administered by continuous infusion into part A of the cannula through a capillary tube. The rate of the infusion was between 0.08 and 0.1 cc. per minute. By directing the dehydroascorbic acid solution into the vigorous stream of oxygen-carbon dioxide mixture bubbling through the fluid in A, it was uniformly distributed in the latter in close proximity to the heart. As the bicarbonate buffer solution in the cannula was replaced at the rate of 2 to 2.5 cc. per minute, the diluted dehydroascorbic acid solution was not exposed to the destructive effects of the alkaline medium for more than a very short time.

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In this way we feel certain that in 4 different experiments the heart for from 2 to 3 hours actually was under the influence of a concentration of dehydroascorbic acid not too far below the calculated values of 1:7,000 to 1:9,000 and higher than any concentrations of dehydroascorbic acid to which the heart could have been exposed under the conditions of the experiments with *l*-ascorbic acid solutions 1:10,000 described above. Under the influence of the dehydroascorbic acid solution the progressive decrease in the height of systolic contraction was more marked than in control experiments, in which bicarbonate buffer solution alone was used, or in which the sodium acetate solution of pH 6 without dehydroascorbic acid was infused. (Further control experiments showed that the continuous infusion of the sodium acetate solution did not interfere with the action of the ascorbic acid solutions.) Apart from the slight negative inotropic effect dehydroascorbic acid caused no significant change. The same result was obtained with the oxidation product of *d*-iso-ascorbic acid. Dehydroascorbic acid, therefore, cannot be responsible for the action of ascorbic acid on the frog heart.

IV. *Hydrogen peroxide, the cause of the heart action of ascorbic acid solutions.* As neither the two ascorbic acids nor their oxidation products in themselves caused the action upon the heart, and as the blocking of the hydroxyl groups within the lactone ring yielded an inert compound, it seemed possible that the heart effect might be due to a side reaction taking place in the solution during the dehydrogenation of ascorbic acid. This thought was supported by Peugnet's finding that copper was essential for the heart action observed by him.

Guzman Barron, DeMeio, and Klemperer studied the catalytic action of copper (Cu^{++}) upon the dehydrogenation of ascorbic acid (13) and, on the basis of observations by Traube (14) and by Wieland and Franke (15), postulated the appearance of hydrogen peroxide in the solution during the reoxidation of the reduced catalyst (Cu^{+}). The presence of hydrogen peroxide in ascorbic acid solutions was actually demonstrated by Holtz and Triem (16) and by Schales (17). As was already mentioned, oxidizing agents among them certain peroxides, were found to have a systolic action upon the isolated heart of the frog (11).

1. If hydrogen peroxide was responsible for the heart action of solutions of *l*-ascorbic acid or *d*-iso-ascorbic acid, then a destruction of hydrogen peroxide immediately upon its formation should prevent the effect. When two drops of frog blood (2 experiments) or 0.05 cc. of a solution of a highly purified catalase preparation (4 experiments) was added to 500 cc. of a 1:10,000 solution of *l*-ascorbic acid, although the chemical analyses indicated that dehydrogenation took place, the characteristic action upon the heart was prevented.

As Holleman first observed, pyruvic acid and hydrogen peroxide react very rapidly to form acetic acid, carbon dioxide, and water (18–20). Lipmann investigated the speed of the reaction at various concentrations of pyruvic acid. At a concentration of 0.1% the rapidity of the reaction was of the same order as that of the destruction of hydrogen peroxide by catalase. Sevag (22) utilized pyruvic acid to protect the enzymatic activity of pneumococci against the destructive effect of hydrogen peroxide, which is formed during the culture of many microörganisms (23). If to an ascorbic acid solution 1:10,000, sodium pyruvate was added in a concentration of 0.1%, the characteristic heart action did not occur, while the decrease in the reducing power of the ascorbic acid solution was not prevented.

2. The appearance of hydrogen peroxide in *l*-ascorbic acid solution has been ascertained qualitatively (16, 17, 24). We attempted an approximate quantitative estimation employing for comparison hydrogen peroxide solutions of known strength in distilled water and using the luminol reaction of Lommel (25). This method is based on the appearance of luminescence when hydrogen peroxide reacts with 3-aminophthal-hydrazide (luminol). The reagent as employed by Schales (24) contained, in 100 cc.: luminol 0.1 gram; mesohemin 0.003 gram; and sodium carbonate 1 gram. Five cubic centimeters were added to 10 cc. of *l*-ascorbic acid solution or to 10 cc. of an appropriate dilution to make it possible to see the luminescence in the dark-room at a distance of 50 cm. from the eye of the observer.

Three experiments with *l*-ascorbic acid and one with *d*-iso-ascorbic acid were made by the usual method but without a frog heart attached to the cannula. Five to ten minutes after preparing the ascorbic acid solution 1:10,000 in bicarbonate buffer solution saturated with oxygen-carbon dioxide mixture, hydrogen peroxide was found in the solution in the Mariotte bottle in a concentration of about 1:10 million. Administration was then started to the cannula where a stream of oxygen-carbon dioxide mixture bubbled through the solution. Within 20 minutes the hydrogen peroxide concentration in the solution in the cannula approached 1:500,000, and 10 minutes later it was found to be 1:250,000. One hour after the beginning of the experiment a concentration of between 1:200,000 and 1:100,000 was reached, and this was maintained for the second hour. After that the concentration began to decrease.

3. The range of concentration of hydrogen peroxide causing the characteristic effect upon the frog heart was established under our experimental conditions in ten experiments. As hydrogen peroxide is not stable in an alkaline medium we administered it in the way described for dehydroascorbic acid, dissolving it in an 0.8% sodium acetate solution of pH 6 and infusing the solution continuously into part A of the cannula at a rate of 0.08 to 0.1 cc. per minute. The actual concentrations reaching the heart were between 1:100,000 and 1:1 million. As in the experiments with ascorbic acid the initial increase in the height of contraction and the final systolic standstill could be observed. An increase in heart rate occurred in some experiments only. While a concentration of 1:500,000 caused systolic standstill within a period of about 3 hours, a range of concentration of hydrogen peroxide between 1:100,000 to 1:300,000 led to systolic standstill within 1 to 2 hours as was observed with ascorbic acid solution of an initial concentration of 1:10,000.

The results of these three series of experiments are consistent with the assumption that the characteristic heart action of ascorbic acid solutions is caused by hydrogen peroxide.

SUMMARY

l-Ascorbic acid or *d*-iso-ascorbic acid, when administered continuously to the isolated frog heart in an initial concentration of 1:10,000, led to an increase in the height of contraction and an inconsistent increase in rate, and caused irreversible systolic standstill within 1 to 2 hours. Our experiments indicate that the effect upon the frog heart was not due to *l*-ascorbic acid or *d*-iso-ascorbic acid itself, nor to their products of dehydrogenation, the corresponding dehydroascorbic acids. The effect was absent when the ascorbic acids were protected against the dehydrogenation by excluding oxygen from the solution, or, in the case of *l*-ascorbic acid, by introducing methyl groups into the hydroxyl groups of the lactone ring to form the dimethyl ascorbic acid. During the process of dehydrogenation of *l*-ascorbic acid or *d*-iso-ascorbic acid 1:10,000 in bicarbonate buffer solution, hydrogen peroxide appeared in the solutions and was found to reach concentrations of the range of 1:200,000 to 1:100,000 under the conditions of our experiments. Evidence is presented which indicates that the characteristic action of *l*-ascorbic acid and of *d*-iso-ascorbic acid solutions upon the frog heart can be accounted for by these concentrations of hydrogen peroxide.

Acknowledgements. The authors wish to express their gratitude to Dr. J. H. Roe for suggesting the method of oxidation of ascorbic acid with Norit in the pesence of acetic acid; to Dr. R. Tyslowitz for making the colorimetric estimations of ascorbic acid; and to Dr. O. Schales for supplying the luminol reagent.

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THE EFFECT OF COCAINE, ERGOTAMINE AND YOHIMBINE ON THE ACTIVITY OF PHENOL SULFUR ESTERASE

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It is generally assumed that the inactivation of epinephrine *in vivo* is an enzymatic process. The enzyme involved is not yet identified, but it is believed to be an oxidase. The review of literature is omitted because of the numerous recent reviews of the subject. That the inactivation of epinephrine is due only to oxidative processes has been questioned recently mainly on the following grounds:

1) The distribution of oxidative enzymes in various tissues does not parallel the ability of the tissues to destroy epinephrine (1–3). The rate of oxidation by the enzymes is probably too slow (4).

2) The degree to which a substance potentiates epinephrine often does not coincide with the degree to which it inhibits oxidative processes. Conversely the degree to which a substance decreases the effect of epinephrine often does not coincide with the degree of increase of the oxidative processes. Affinity to oxidative enzymes and biological effect often differs significantly with some of the epinephrine-like substances (5).

3) Clark and Raventos (6) calculated that only 50% of the inactivation of epinephrine *in vivo* could be attributed to the action of oxidative enzymes.

In 1940 Richter (3, 7) found that epinephrine administered by mouth can be recovered from the urine as a sulfur ester. This indicates that a sulfatase plays a part in the inactivation of epinephrine. Among the known enzymes, phenol sulfur esterase is probably the one to which this process could be attributed.

This paper concerns itself with the effect of cocaine, ergotamine and yohimbine on the activity of the phenol sulfur esterase to determine whether or not this effect parallels their influence on the effect of epinephrine *in vivo*. Our work does not give a final answer to the question of whether or not phenol sulfur esterase is involved in the inactivation of epinephrine, but suggests that it may be.

Methods. The enzyme activity of phenol sulfur esterase was investigated *in vitro*.

The enzyme was obtained from fresh cat muscle prepared according to the method described by Neuberg (see 8). 1.25% potassium phenol sulfonate was used as substrate. This salt was prepared according to the method of Verley and Czepek (see 8).

Two cc. of muscle extract, 8 cc. of the substrate, 500 mgm. of calcium carbonate and 1 drop of toluol were incubated for 3 days at 30°C. To some samples of this mixture cocaine, ergotamine tartrate or yohimbine were added before incubation to make a concentration of 5 per cent. The activity of the phenol sulfur esterase was measured by the amount of phenol liberated. Because phenol is very soluble in toluol, the toluol content of the samples was kept at a minimum. Large amounts of calcium carbonate were added, first to speed

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ity occurs at pH 5–8.[16] Solubility in water is only 22 mg per 100 ml and is somewhat higher in ethanol. Biotin is insoluble in most organic solvents.

Vitamin C Ascorbic acid is a white, crystalline substance existing in nature both as the reduced and the oxidized form—dehydroascorbic acid. Biological activity is found in the L-isomer although D-glucoascorbic and other analogs have slight activity. Ascorbic acid is available commercially as the L-acid and as the sodium salt. It is a moderately strong reducing agent; redox potential +.127v at pH 5. Crystalline ascorbic acid can be stored under normal laboratory conditions for years with little change in activity. Its oxidation is catalyzed by traces of copper and iron. In the absence of oxygen and other oxidizing agents it is heat stable. The copper-catalyzed oxidation of ascorbic acid is a first order reaction in an oxygen saturated solution. Under these conditions stability is superior at pH 3–4.5 compared to pH 6.0–7.0 (Table 2).[17] Degradation of ascorbic acid in concentrated solutions or under anaerobic conditions has lower velocity constants than shown in Table 2 and different pH stability optima.

TABLE 2
Ascorbic Acid Oxidation Kinetics, Cu^{++} 2.8 ppm[17]

Buffer	*pH*	$k_1 \times 10^4$ (min^{-1}) 25°C *l-Ascorbic Acid*
$H_3PO_4 + Na_2HPO_4$	3.0	120.0
	3.5	250.0
	4.0	530.0
$KH_2PO_4 + Na_2HPO_4$	4.5	955.0
	5.0	1660.0
	5.5	2560.0
	6.0	3340.0
	6.5	2880.0
	7.0	2800.0
	7.5	2860.0
	8.0	2900.0

In propylene glycol, maximum stability is reported to occur at pH 6.0–7.0.[10] Oxidation at alkaline pH is not metal catalyzed and is very rapid. The oxidation to dehydroascorbic acid is reversible; the oxidation of dehydroascorbic acid to 2,3 diketo-L-gluconic acid is not reversible. Plant tissues, with the exception of milk and liver, are the only foods which have an appreciable ascorbic acid content. Citrus fruits, tomatoes, potatoes and cabbage are the best dietary sources.

Fat Soluble Vitamins

Vitamin A In nature vitamin A occurs in several forms—vitamin A alcohol (retinol), vitamin A aldehyde (retinal), vitamin A acid (retinoic acid) and vitamin A palmitate (retinyl palmitate). Vitamin A palmitate predominates since it is the primary liver storage form. Vitamin A_1 in the all trans form has a biological activity defined as 0.300 $\mu g = 1$ international unit (I.U.).

THE OXIDATION OF ASCORBIC ACID BY OXIDIZED ADRENALIN AND CYTOCHROME *c**

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Previous experimental results suggest that adrenalin and ascorbic acid are linked to a system of reductions and oxidations which play an important rôle in the formation of the aqueous humor (1). In order to obtain information about the enzymatic basis of these reactions we have investigated the distribution of some of the known oxidative enzymes in the tissue components of the ciliary body and of the chorioid plexus (2). It is possible, however, that the interaction of some of the presumptive mediators might be subject to an enzymatic catalysis that had so far escaped notice. In this connection we have examined the effect of various tissue preparations upon the reaction between ascorbic acid and oxidized adrenalin. We found that in the presence of the latter the disappearance of ascorbic acid was greatly accelerated and the oxygen uptake considerably enhanced by the addition of cytochrome *c*.

It has been generally accepted that cytochrome *c* accelerates the oxidation of various substrates by molecular oxygen only in systems which contain cytochrome oxidase. Our experiments suggested that an increase in the rate of oxidation of ascorbic acid by cytochrome *c* can also occur when oxidized adrenalin is added as the autoxidizable component instead of cytochrome oxidase. This possibility was studied in greater detail and the results are reported in this paper.

EXPERIMENTAL

For the oxidation of adrenalin we prepared a solution which contained 1 mg. of a crystalline preparation (Parke, Davis) in 0.5 ml. of a 0.05 M phosphate buffer at pH 7.2. Between 2.0 and 3.5 ml. of this solution were pipetted into a Warburg flask and shaken at a rate of about 80 oscillations per minute. Oxygen uptake starts after a lag period of 1 to 2 hours, depending upon the purity and the age of the adrenalin preparation. At the same time the formation of red oxidation products can be observed. If not stated otherwise, we allowed the oxygen uptake to proceed until 80 to 110 c.mm. of oxygen had been consumed per mg. of adrenalin, which

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required 4 to 6 hours at a temperature of 37.5°. We assume that the solution contains after this time some unoxidized adrenalin and several oxidation products at different steps towards the maximum possible oxidation. As a matter of convenience and because of lack of better information, this mixture will be called oxidized adrenalin. Further comments concerning the oxidation products of adrenalin will be given in the discussion.

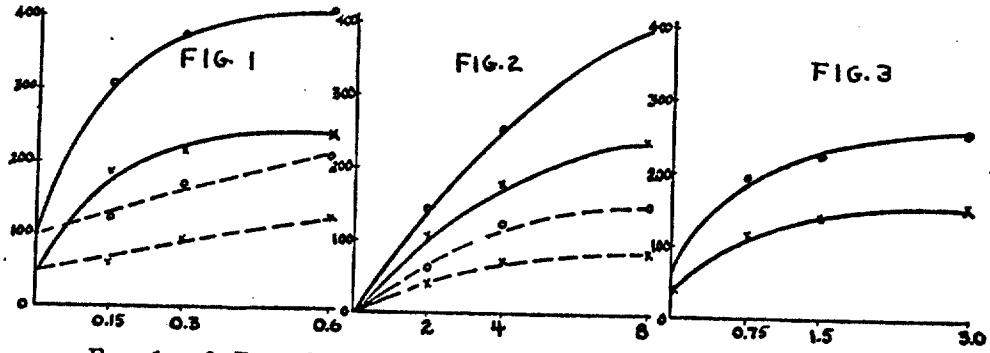
The rate of the oxidation of ascorbic acid was determined by measuring the oxygen uptake manometrically or by titrating the ascorbic acid with dichlorophenol indophenol. For the manometric determination the solutions of ascorbic acid or of the substances which were used in its place were pipetted into the side arms of Warburg flasks, while the other components of the system were pipetted into the main compartment. The content of the side arm was tipped into the main compartment after thermoequilibration, and readings were taken every 5 minutes at 37.5° with air as the gas phase. It was found to be essential to shake the manometers at a high oscillation rate of about 120 per minute. All solutions were made up in 0.05 M phosphate buffer of pH 7.2, and the same buffer solution was used for the adjustment to 3.0 ml. of the fluid volume in the Warburg flasks. For all purposes we took double distilled water, with the second distillation carried out in an all-glass still.

The titrations of ascorbic acid were carried out by transferring 0.1 or 0.2 ml. portions of the content of the Warburg vessels into 50 ml. beakers which contained 1.0 ml. of a 5 per cent solution of metaphosphoric acid. A slight opalescence developed when cytochrome *c* was present in the samples. With a little practice, we had no difficulty in recognizing the end-point of the titration in samples which showed this slight turbidity or the pale color due to the presence of cytochrome *c* or of oxidized adrenalin. The solution of dichlorophenol indophenol contained 72 mg. of the dye in 100 ml. of distilled water. In one series of experiments the rate of disappearance of ascorbic acid was determined only by titration. In this instance we used 50 ml. Erlenmeyer flasks instead of the Warburg flasks, since it is easier to remove aliquots from the solution while the reaction is in progress.

Results

The acceleration of the oxidation of ascorbic acid by cytochrome *c* in the presence of oxidized adrenalin is demonstrated in Figs. 1 to 3. The rate of autoxidation of ascorbic acid is indicated in Figs. 1 and 3 by the values for the oxygen uptake at the intersection of the curves with the ordinate axis. It can be seen that oxidized adrenalin itself slightly accelerates the oxidation of ascorbic acid. Addition of cytochrome *c* enhances this acceleration up to 9 times, depending upon the concentration of the

components of the system. With ascorbic acid the increase in the rate of oxidation follows the increase in concentration up to the highest observed concentration (1.5×10^{-2} M) and diminishes only slightly in the higher range (Fig. 2). In the case of cytochrome *c* and of oxidized adrenalin the acceleration reaches a more definite limiting value. The concentration of cytochrome *c* at this point is 3.1×10^{-5} M. Since it is not known which of the oxidation products of adrenalin are active in our system, a valid statement about the concentrations involved cannot be made. If one would assume that all the adrenalin has been transformed into active material, the concentration would correspond to 1.1×10^{-3} M. Probably it is much below this figure. The observed effect is greatly dependent



FIGS. 1 TO 3. Dependence of the oxygen uptake (c.mm. in 20 minutes, ordinate scale), in a system containing oxidized adrenalin, ascorbic acid, and cytochrome *c*, upon the concentration of the components. All figures refer to the content per flask; final fluid volume, 3.0 ml. of 0.05 M phosphate buffer, pH 7.2. Fig. 1, 6.0 mg. of ascorbic acid; 3.0 mg. of cytochrome *c*; abscissa, ml. of solution of oxidized adrenalin. Fig. 2, 0.2 ml. of solution of oxidized adrenalin; 3.0 mg. of cytochrome *c*; abscissa, mg. of ascorbic acid. Fig. 3, 0.2 ml. of solution of oxidized adrenalin; 3.0 mg. of ascorbic acid; abscissa, mg. of cytochrome *c*.

upon the pH. Our experiments were carried out at pH 7.0 to 7.5. At higher alkalinity measurements become less reliable because of the increasing autoxidation of ascorbic acid. When the acidity is increased, the effect is diminished and is practically absent at pH 5.5.

In order to exclude the possibility that an unknown amount of the oxidized adrenalin directly oxidizes the ascorbic acid, we placed oxidized adrenalin, corresponding to 1 mg. of adrenalin, and 3 mg. of cytochrome *c* in the main compartment of a Warburg vessel and 6 mg. of ascorbic acid in the side arm. The vessel was flushed for 10 minutes with nitrogen and after a further equilibration period of 5 minutes the ascorbic acid was tilted into the main compartment. After rapid shaking for 20 minutes ascorbic acid was determined in an aliquot by titration with dye. We could detect

no measurable disappearance of ascorbic acid. It follows that the presence of oxygen is essential for the reaction. The values obtained manometrically for the oxygen uptake after 20 minutes with different concentrations of oxidized adrenalin (Table I) were compared with the data for the disappearance of ascorbic acid as determined by titration with dichlorophenol indophenol. The titrations were carried out with aliquots of the same samples that were used for the manometric determinations. The dye titration measures only the first step in the oxidation of ascorbic acid, while the manometric values indicate the total oxygen consumption including the oxidations beyond dehydroascorbic acid. In the presence of cytochrome *c* the values obtained both manometrically and by dye titration decrease only by about 24 per cent (Column I) and 10 per cent (Column III) respectively with decreasing concentration of oxidized adrenalin.

TABLE I

Comparison of the Effect of Cytochrome C upon Oxidation of Ascorbic Acid As Determined Manometrically and by Dye Titration

Content per flask, cytochrome *c* 3 mg., ascorbic acid 6 mg.; final fluid volume, 3.0 ml. of 0.05 M phosphate buffer, pH 7.2; reaction time, 20 minutes.

Solution of oxidized adrenalin corresponding to adrenalin content of 2 mg. in 1.0 ml.	Oxygen uptake in mM × 10^{-3}; manometric determination			Ascorbic acid disappearance in mM × 10^{-3}; dye titration				
	Cytochrome *c*		I/II	Cytochrome *c*		(III)/(IV)	(I)/(III)	(II)/(IV)
	+ (I)	− (II)		+ (III)	− (IV)			
ml.								
0.6	1.82	0.94	1.94	2.17	1.15	1.89	0.84	0.82
0.3	1.64	0.74	2.22	2.16	1.18	1.83	0.76	0.63
0.15	1.38	0.41	3.37	1.95	0.86	2.26	0.71	0.48

Of the same order of magnitude (25 per cent) is the decrease in the absence of cytochrome *c* when the values are determined by dye titration (Column IV), while a much greater decrement (54 per cent) is observed in the manometric data (Column II), corresponding to an equally marked increase in the ratio of Column I to II. Calculation of the molecular ratios between oxygen consumption and ascorbic acid disappearance shows that at the highest concentration of oxidized adrenalin considerably more than 1 atom of oxygen is used per molecule of ascorbic acid. The oxygen uptake in excess of 0.5 M O_2 per 1 M ascorbic acid is maintained at a high level by the added cytochrome *c* but decreases rapidly with decreasing concentrations of oxidized adrenalin in the absence of cytochrome *c* (Column II/IV). The results show that cytochrome *c* accelerates not only the first step in the oxidation of ascorbic acid but also the oxygen uptake of more than 1 atom of oxygen per molecule of ascorbic acid.

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upon Oxidation of Ascorbic Acid As
and by Dye Titration

ascorbic acid 6 mg.; final fluid volume,
reaction time, 20 minutes.

orbic acid disappearance in mM × 10^{-4}; dye titration				
Cyto c		(III)/(IV)	(I)/(III)	(II)/(IV)
(I)/(III)	(II)/(IV)			
.17	1.15	1.89	0.84	0.82
.16	1.18	1.83	0.76	0.63
.95	0.86	2.26	0.71	0.48

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In order to obtain some information about the nature of the effect we tested several substitutes for each one of the three reacting substances.

Cytochrome c—In replacing cytochrome *c* we were interested to find out whether the effect could be produced with metals in the form of ions or bound in complex molecules of the type of cytochrome *c*. We added copper as cupric sulfate in a final concentration of from 0.25 to 2.5 × 10^{-4} M. The higher concentrations considerably accelerated the oxidation of ascorbic acid, but the acceleration was the same in the presence and absence of oxidized adrenalin. Using ferric chloride in the same concentrations we observed no acceleration of the autoxidation of ascorbic acid and the reaction was not catalyzed in the presence of oxidized adrenalin. Among the substances which are more closely related to cytochrome *c* we tested a crude preparation of hemoglobin. Oxalated human blood was washed in the centrifuge with physiological saline until the supernatant was free from oxalate and 1 ml. of the cell sediment was suspended in 10 ml. of distilled water. After completion of the hemolysis the ghost cells were centrifuged off and the supernatant was used as "hemoglobin" solution in these experiments. The concentration of the hemolysate (protein nitrogen) was adjusted to equal the concentration of the cytochrome *c* solution in our standard procedure. Under these conditions the reaction between oxidized adrenalin and ascorbic acid was not accelerated by the hemoglobin in the solution.

Oxidized Adrenalin—In a larger series of experiments we tried to obtain some information about the activity of adrenalin derivatives at different states of oxidation. The derivatives were produced by autoxidizing the adrenalin for different lengths of time in the usual way in Warburg flasks. The oxidation was followed manometrically and solutions were tested after the oxygen uptake had proceeded to the desired value. We obtained about half of the usual activity after an oxygen uptake of about 30 c.mm. per mg. of adrenalin and the full effect could be observed in most samples after an oxygen uptake of 50 c.mm. per mg. of adrenalin. These figures would correspond respectively to an oxidation of about 1 and 1.6 equivalents if one assumes that the oxidation progresses uniformly. The appearance of the red color, however, indicates that some of the adrenalin has undergone at least 4 equivalents of oxidation and, hence, that a part of the adrenalin must still be present in its original reduced state. Better clarification of this question will have to await the preparation of the various derivatives.

The compounds which were tested as substitutes for oxidized adrenalin were the oxidation products of hydroquinone, catechol, dihydroxyphenylalanine, and butanefrine[1] (terminal methyl group in adrenalin substituted

[1] Winthrop Chemical Company, Inc.

by butyl radical). These derivatives were obtained also by autoxidation in the same way as is described for adrenalin. Since the autoxidation of the first three substances at pH 7 proceeded too slowly to give the required oxygen uptake within a reasonable time, we used here pH 9.0, after having convinced ourselves that the oxidation products of adrenalin obtained at this pH have the same activity as those obtained at pH 7. We tested the solutions at two states of oxidation of the four compounds, corresponding to an oxygen uptake of 80 and 140 c.mm. per mg. of the respective substance. The oxidation products of hydroquinone, catechol, and dopa were completely ineffective. With dopa a precipitate formed in the presence of cytochrome *c* and it cannot be excluded that the failure in this case was due to the elimination of dopa from the solution. The oxidation product of butanefrine, on the other hand, was found to be only 20 per cent less active than oxidized adrenalin itself.

Ascorbic Acid—It was attempted to substitute for ascorbic acid other substances which are readily oxidized and, in turn, are active reducing agents, especially for the reduction of cytochrome *c*. We tested adrenalin itself as well as hydroquinone, catechol, and cysteine, substances which are frequently used as substrates in the cytochrome system. Cysteine was not only not oxidized but it even acted as an inhibitor for the catalyzed oxidation of ascorbic acid. With catechol, adrenalin, and hydroquione no extra oxygen uptake was observed above the slow rate of autoxidation of these compounds. Isoascorbic acid is as effective as ascorbic acid but with glucose no O_2 uptake was observed.

Inhibitions of Cytochrome c Effect—The effect was found to be sensitive to cyanide. In a concentration of 3.5×10^{-3} M the oxygen uptake was practically completely eliminated; with a concentration of about 1.0×10^{-3} M we obtained a decrease of about 50 per cent. The inhibitory action of cysteine has been mentioned in a previous paragraph. A complete inhibition was obtained at a concentration of 5.0×10^{-3} M and an inhibition of 20 per cent was obtained with 7.5×10^{-4} M. Two substances with pharmacological action synergistic to adrenalin, ephedrine and 2-methyltetrahydroisoquinoline hydrochloride (3), were also tested. 2 mg. of one or the other of these were added to the usual mixture in Warburg flasks. This amount was 5-fold of the amount of adrenalin present in these experiments, but no inhibitory or potentiating action could be detected.

We were interested in the effect of oxidized ascorbic acid on the cytochrome *c* effect. Ascorbic acid was oxidized by bubbling air through a solution of this substance at pH 8.0. The disappearance of reduced ascorbic acid was determined by dye titration. The sample was used when half of the ascorbic acid was present in oxidized form, the other

ves were obtained also by autoxidation
or adrenalin. Since the autoxidation of
roceeded too slowly to give the required
time, we used here pH 9.0, after having
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d 140 c.mm. per mg. of the respective
s of hydroquinone, catechol, and dopa
dopa a precipitate formed in the pres-
ot be excluded that the failure in this
dopa from the solution. The oxidation
hand, was found to be only 20 per cent
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d to substitute for ascorbic acid other
ized and, in turn, are active reducing
of cytochrome c. We tested adrenalin
echol, and cysteine, substances which
in the cytochrome system. Cysteine
n acted as an inhibitor for the catalyzed
cat l, adrenalin, and hydroquione
ed e the slow rate of autoxidation
acid is as effective as ascorbic acid but
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—The effect was found to be sensitive
3.5 × 10^{-3} M the oxygen uptake was
with a concentration of about 1.0 ×
f about 50 per cent. The inhibitory
d in a previous paragraph. A complete
tration of 5.0 × 10^{-3} M and an inhibi-
h 7.5 × 10^{-4} M. Two substances with
to adrenalin, ephedrine and 2-methyl-
de (3), were also tested. 2 mg. of one
o the usual mixture in Warburg flasks.
amount of adrenalin present in these
otentiating action could be detected.
of oxidized ascorbic acid on the cyto-
s oxidized by bubbling air through a
8.0. The disappearance of reduced
dye titration. The sample was used
s present in oxidized form, the other

half in reduced form. To a control sample we added the same amount of reduced ascorbic acid without oxidized products. When tested in our system, the same acceleration of the disappearance of ascorbic acid by cytochrome *c* and oxidized adrenalin was observed in both samples. The presence of oxidized ascorbic acid has no inhibitory effect on this reaction.

DISCUSSION

The results of our experiments demonstrate that the addition of a cytochrome *c* preparation to a solution which contains oxidized adrenalin and ascorbic acid causes a considerable increase of the oxygen uptake and of the rate of disappearance of ascorbic acid. In discussing the nature of this effect the question arises as to the purity of our cytochrome *c* preparation. In consideration of the starting material from which the cytochrome *c* is obtained (hog hearts) it could be objected that the increased oxidation of ascorbic acid was caused by an incomplete elimination of cytochrome oxidase or by the presence of metal ions, especially of copper. In respect to the first possibility we should like to point out that the preparation of cytochrome *c* involves a precipitation by trichloroacetic acid and by acetone, which makes it very unlikely that any activity of the unstable cytochrome oxidase has remained. Moreover, no increase in the rate of oxidation of ascorbic acid could be discovered when a solution of cytochrome *c* was added to ascorbic acid in the absence of oxidized adrenalin. This latter result demonstrates more directly the absence of cytochrome oxidase in our preparations and differentiates the effect from the catalytic ascorbic acid autoxidation by various hematoporphyrins (4). It shows in addition that our cytochrome *c* preparation cannot contain active metal ions. We arrived at the same conclusion from results of experiments in which the cytochrome *c* was replaced by metal ions. Copper (as cupric sulfate) actually caused an increase in the rate of autoxidation of ascorbic acid. However, the increase was the same in the presence and in the absence of oxidized adrenalin. Ferric ions added in the same quantity as cupric ions did not cause a significant increase of the autoxidation, either in the presence or in the absence of oxidized adrenalin. Equally ineffective was a hemolysate which contains iron both bound to the porphyrin ring of hemoglobin and also as the usual ionic contamination.

Although the argument above makes it seem unlikely that the effect of our cytochrome *c* preparation is due to the presence of free metal ions or of cytochrome oxidase, our results with inhibitors would seem to indicate that a metal in some form is involved, since cyanide and cysteine, which are typical metal binders, act as strong inhibitors. Recent spectroscopic investigations suggest that cyanide can combine with cytochrome *c*, and therefore a participation of the porphyrin iron in cytochrome *c* has to be

considered (5). A possible rôle of H_2O_2 which has been found to be formed in metal-catalyzed autoxidations of ascorbic acid cannot be excluded at the present time (6).

As an explanation of the accelerated oxidation of ascorbic acid one might be inclined to assume that the cytochrome *c* in our preparation is rapidly oxidized by one of the oxidative derivatives of adrenalin and is reduced by ascorbic acid. However, adrenochrome and other related compounds have a lower oxidation-reduction potential than cytochrome *c* itself, and these compounds could hardly act as efficient oxidizing agents for cytochrome *c*. Moreover, with cytochrome *c* as mediator it should be possible to replace ascorbic acid by any other substance which would reduce cytochrome *c*. Our results, however, indicate that hydroquinone, catechol, or adrenalin itself, very effective reducing agents for cytochrome *c* in the conventional cytochrome oxidase system, cannot act as substitutes for ascorbic acid in our experiments. This specificity of the reducing agent is striking, and it could mean that the reaction depends on the combination of oxidized adrenalin and ascorbic acid with a direct electron exchange between these compounds, the catalyst acting as an adsorbing surface which would bring the two substances into closer proximity and enhance thereby the probability of their interaction. Whatever the rôle of the catalyst may be, oxidized adrenalin seems to have a higher affinity than ascorbic acid, since the saturation concentration for the former is considerably lower than for the latter.

A connection between this effect and the physiological function of either adrenalin or ascorbic acid cannot be seen at the present time. In particular it should be pointed out that the compounds with adrenergic activity (ephedrine, isoquinoline) are without effect on the acceleration. On the other hand it should be kept in mind that similar mechanisms could play a rôle in the case of other physiological quinones, such as vitamin K.

SUMMARY

1. The rate of ascorbic acid oxidation in the presence of oxidized adrenalin is markedly enhanced by addition of cytochrome *c*.

2. The effect is specific in that cytochrome *c* cannot be replaced by copper or iron ions or by hemoglobin. Oxidized adrenalin cannot be replaced by oxidized hydroquinone or catechol and ascorbic acid cannot be replaced by such reducing agents as hydroquinone, catechol, cysteine, or glucose, while isoascorbic acid is equally effective.

3. The reaction is inhibited by cyanide and cysteine. Ephedrine, isoquinoline derivatives, and oxidized ascorbic acid have no inhibitory effect.

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On the isolation of an ascorbic acid-dependent DPNH-oxidase

In the further course of our investigations of the effect of ascorbic acid in the adrenal glands (1,2,3,4) we isolated an enzyme system from the adrenal glands which can dehydrate DPNH- in the presence of ascorbic acid and O2. This enzyme system is localized in the microsomes.

It can be precipitated from the supernatant portion of a homogenate of the adrenal glands of the hog after removal of the mitochondria, or directly from the adrenal gland microsomes with ammonium sulfate at a saturation concentration of 40%. After the washed crude product has been treated with 10% alkaline desoxycholate solution we obtain an absorption spectrum with the following absorption maxima:

oxidized: 411 m mu
reduced: 423 m mu; 526 m mu; 557 m mu (with Na2S204).

The absorption maxima are identical with those of the cytochrome b5, which was recently isolated from rat liver microsomes. (5, 6). According to our experimental results, the enzyme system permits the direct oxidation of DPNH by means of ascorbic acid

DPNH --- enzyme system (cyt.b5) ---- FE+++

where Fe+++ is reduced to Fe++ and serves thus as an electron acceptor. This reaction (2) is faster than (1). Since ascorbic acid can only accept electrons as monohydroascorbic acid, the transition of ascorbic acid into the monohydroform is velocity-determinant in the reaction (1) with O2.

The protein complex still has a slight cytochrome c-oxidase-effect, which is completely eliminated by KCN, while the reaction (1) and (2) are not influenced by the addition of KCN.10^{-3} mole or sodium azide.10^{-3} mole.

For the presence of effective SH-groups in the enzyme system argues the fact that p-chloromercury benzoate inhibits the reactions (1) and (2). As other inhibitors are known so far Ca++ and Mn++. The metal inhibition can be partly eliminated by the addition of versene Mg++ and antimycin A

do not inhibit. TPNH is not oxidized by the present protein complex under the given conditions.

Ascorbic acid can be replaced by d-iso ascorbic acid, but not by gluthathion or adrenalin. d-iso ascorbic acid is not as effective,however, as ascorbic acid itself.

As we reported earlier, the DPNH oxidation in the adrenal gland, mitochrondrial is accelerated by ascorbic acid. Whether the enzyme system described here also occurs in the mitochondria or whether dragged-in microsomes cause the effect is still being investigated. We are also investigating to what extent the enzyme system occurs in other organisms. So far the above described effect (reaction 1) has also been found in the liver microsomes of the rat.

At present we are engaged in purifying the present protein fraction further in order to expand the experimental findings obtained so far in quantitative respect, about which we will then report in detail.

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(Translated by Carl Demrick Associates,Inc/IE/t)

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Über die Isolierung einer Ascorbinsäure-abhängigen DPNH-Oxydase

Im weiteren Verlaufe unserer Arbeiten über die Wirkung der Ascorbinsäure in der Nebenniere[1,2,3,4] haben wir aus Nebennieren ein Enzymsystem isoliert, das in Gegenwart von Ascorbinsäure und O_2 DPNH dehydrieren kann. Dieses Enzymsystem ist in den Mikrosomen lokalisiert.

Es lässt sich aus dem Überstand eines Schweinenebennierenhomogenates nach Entfernung der Mitochondrien oder aus Nebennierenmikrosomen direkt mit Ammoniumsulfat bei einer Sättigungskonzentration von 40% ausfällen. Nach Behandlung des gewaschenen Rohproduktes mit 10%iger alkalischer Desoxycholatlösung erhält man ein Absorptionsspektrum mit folgenden Absorptionsmaxima:

Oxydiert: 411 mμ
reduziert: 423 mμ; 526 mμ; 557 mμ.
(mit $Na_2S_2O_4$)

Die Absorptionsmaxima sind identisch mit denen des Cytochrom b_5, das vor kurzem aus Rattenlebermikrosomen isoliert wurde[5,6]. Nach unseren experimentell gewonnenen Ergebnissen ermöglicht das Enzymsystem die direkte Oxydation von DPNH unter Vermittlung der Ascorbinsäure

$$\text{DPNH} \longrightarrow \underset{(\text{Cyt } b_5)}{\text{Enzymsystem}} \longrightarrow \text{(Monodehydro)ascorbinsäure} \longrightarrow O_2 \qquad (1)$$

Setzt man an Stelle von Ascorbinsäure und O_2 Fe^{+++} in komplexer Form ein, so verläuft die Reaktion

$$\text{DPNH} \longrightarrow \underset{(\text{Cyt } b_5)}{\text{Enzymsystem}} \longrightarrow Fe^{+++} \qquad (2)$$

wobei Fe^{+++} zu Fe^{++} reduziert wird und somit als Endelektronenacceptor dient. Diese Reaktion (2) verläuft schneller als (1). Da Ascorbinsäure nur als Monodehydroascorbinsäure Elektronen aufnehmen kann, ist der Übergang von Ascorbinsäure in die Monodehydroform bei der Reaktion (1) mit O_2 geschwindigkeitsbestimmend.

Der Proteinkomplex hat noch geringe Cytochrom *c*-Oxydasewirkung, die durch KCN vollständig gehemmt wird, während die Reaktionen (1) und (2) durch Zusatz von KCN 10^{-3} mol oder auch Natriumazid 10^{-3} mol nicht beeinflusst werden.

Für die Anwesenheit wirksamer SH-Gruppen im Enzymsystem spricht die Tatsache, dass *p*-Chlormercuribenzoat die Reaktionen (1) und (2) hemmt. Als weitere Inhibitoren kennen wir bis jetzt Ca^{++} und Mn^{++}. Die Metallhemmung lässt sich durch Zusatz von Versen teilweise aufheben. Mg^{++} und Antimycin A hemmen nicht. TPNH wird von dem vorliegenden Proteinkomplex unter den gegebenen Bedingungen nicht oxydiert.

Die Ascorbinsäure lässt sich ersetzen durch *d-iso*-Ascorbinsäure, nicht dagegen durch Glutathion oder Adrenalin. *d-iso*-Ascorbinsäure ist jedoch nicht so wirksam wie die Ascorbinsäure selbst.

Wie wir bereits früher berichtet haben, wird die DPNH-Oxydation in Nebennieren-Mitochondrien durch Ascorbinsäure beschleunigt. Ob das hier beschriebene Enzymsystem auch in den Mitochondrien vorkommt oder ob mitgeschleppte Mikrosomen die Wirkung verursachen, wird noch untersucht. Es wird weiter von uns geprüft, inwieweit das Enzymsystem in anderen Organen vorkommt. Bis jetzt haben wir die beschriebene Wirkung (Reaktion (1)) noch in Lebermikrosomen von Ratten festgestellt.

Wir sind zur Zeit damit beschäftigt, die vorliegende Proteinfraktion weiter zu reinigen, um unsere bisher gewonnenen experimentellen Befunde, über die wir dann im Zusammenhang ausführlich berichten werden, in quantitativer Hinsicht zu erweitern.

Der deutschen Forschungsgemeinschaft danken wir für die Mittel zur Durchführung dieser Arbeit.

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On the mechanism of action of ascorbic acid

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With 4 figures in the text.

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In agreement with Wettstein and coworkers (7) we observed in numerous tests the activating influence of ascorbic acid on the entire corticosteroid biosynthesis and on the 11 beta-hydroxylation in vitro (17). From these tests arose the question regarding the mechanism of action of ascorbic acid particularly in the adrenal glands and in the animal tissue in general.

We suspected that the action of ascorbic acid is based on the activation of the hydrogen-or electron transport in the cell. Starting from this hypothesis, we observed quantitavely the course of the DPNH dehydration[1] in the presence of adrenal gland-mitochondria after addition of ascorbic acid by means of the optical test. As reported earlier (12), ascorbic acid has in this system an accelerating effect on the DPNH-dehydration. Similar observations were made by a number of other authors in tests with vegetable enzyme systems.

Since dehydroascorbic acid shows no effect in the same tests, the further question arose how the hydrogen transportation can be effected by ascorbic acid.

As we assumed in agreement with other authors (1, 9), the hydrogen acceptor proper is a radical-type "monodehydro-ascorbic acid". Since such a radical could not be demonstrated directly heretofore we tried to support our assumption indirectly (10).

On the basis of the above described findings we wanted to check if ascorbic

acid is contained in the mitochondria themselves. In order to obtain further information about the mechanism of action of ascorbic acid, we continued the investigation of the effect of ascorbic acid on the DPNH dehydration with the optical test. We also tried to show the significance of ascorbic acid for vesicular breathing, by measuring the oxygen consumption in the Warburg apparatus. Furthermore we investigated to what extent ascorbic acid can take over hydrogen from DPNH.

I. Determination of the ascorbic acid content of adrenal gland mitochondria.

If ascorbic acid has a function in the transportation of water in cellular breathing, it would have to be contained in the mitochondria, like the other members of the breathing chain, e.g. cytochrome C, DPN etc. Histochemical findings (2) indicate that ascorbic acid occurs in mitochondria, but no exact data about the location and amount of ascorbic acid in mitochondria are possible with these investigation methods.(3). We investigated therefore the adrenal gland mitochondria of the pig, used by us in all experiments, for their ascorbic acid content by paper chromatography (18). In order to make sure that the ascorbic acid was not dragged in during the operation, we washed the mitochondria 1-3 times with the 10-fold amount of isotonic saccharose solution.

Table 1. Ascorbic acid content in the adrenal gland mitochondria.

Mitochondria of g fresh adrenal glands	mg a.a. in mitochondria of 1 g fresh adrenal glands	mg. a.a. related to 1 g mitochondria, dry weight
washed one	0.030	0.60
washed twice	0.015	0.30
washed three times	0.013	0.26

The ascorbic acid content of the adrenal glands of the pig is on the average 200 mg% (6). Of these about 1% are located in the mitochondria, as it results from table 1, and are apparently firmly bonded.

II. Investigations of the effect of ascorbic acid on the hydrogen transport.

As reported (12), we had shown in previous investigations with the optical test that DPNH is oxidized faster by adrenal gland mitochondria in the presence of ascorbic acid and cytochrome C + ascorbic acid than without the above mentioned activators. Still to be proven, however was that DPNH passes over quantitatively into DPN and that ascorbic acid takes over the hydrogen from DPNH. To this end the DPN formed at the end of the optical test was reduced enzymatically to DPNH, and the ascorbic acid was determined by paper chromatography. In order to obtain an indication whether the action of the ascorbic acid is an enzymatic process, we tried to determine a saturation concentration of the ascorbic acid.

For the clarification of the question to what extent the observed effect is specific for L-ascorbic acid, we measured corresponding batches with isoascorbic acid (= D-araboascorbic acid) in the optical test.

1. DPNH dehydration.

a. Experimental part. Adrenal glands of the pig were obtained, as described above (12) according to the method by Hogeboom, Schneider and Pallade (4) and Schneider (19) by fractioned centrifuging in 0.88 m saccharose solution and washed with 0.25 m saccharose. The saccharose solutions contain versene (1.10^{-3} m).

Fig. 1. Effect of ascorbic acid and cytochrome C on the DPNH-dehydration by adrenal gland mitochondria. In 10 ml Krebs-Ringer phosphate buffer: 1 mu mole ATP, 16 mu moles versene. 1. DPNH 0.7 mu mole + ascorbic acid 10 mu moles + cytochrome C 0.01 mu mole; 2. DPNH + mitochondria of 100 g adrenal glands; 3. like 2 + ascorbic acid 10 mu moles; 4 like 2 + cytochrome; 5. like 3 + cytochrome + ascorbic acid. Arrow a: addition of ascorbic acid and cytochrome; arrow b; addition of alcohol and alcohol dehydrogenase, Ordinate: extinction at 340 mu; abscissa: time.

Mitochondria of 20 g adrenal glands are taken up in Krebs-Ringer-phosphate- or Soerensen-buffer of pH 6.8. The suspension thus obtained is filled up to 20 ml. The pH-value of this parent suspension is then pH 6.6 - 6.7. The mitochondria content of 1 ml parent suspension corresponds to 1 g adrenal glands (fresh weight). The measuring solution for the optical test contains the mitochondria of 0.04 - 0.1 g adrenal glands versene ($1.6 \cdot 10^{-3}$ m) and ATP ($0.5 \cdot 10^{-4}$m) and the additions in a total of 10 ml buffer. All solutions, reagents and additions are kept ice-cold until the measurement starts.

After the preparation, the batches are shaken vigorously and the extinction course at 37 deg. C is observed in the Zeiss spectral photometer in 1 cm - cuvettes at 340 m mu. The drop of the extinction of the mitochondria must be determined each time and is eliminated in the graphic representation.

b. Test results. Fig. 1 shows the typical course of an optical test. The pure substances DPNH-cytochrome-ascorbic acid do not react with each other (curve 1). Adrenal gland mitochondria effect only a minor dehydration of DPNH in the concentration used (curve 2) which is accelerated by the addition of ascorbic acid (curve 3). Added cytochrome C has likewise an activating effect on the oxidation of DPNH (curve 4). The effect can be further increased by ascorbic acid (curve 5). The DPNH charge is completely used up after about 20 minutes. In a subsequent enzymatic reduction we obtain in all batches the extinction which corresponds to the DPNH used. This shows that DPNH is only dehydrated and not changed at another point.

2. Hydrogen transport function of ascorbic acid.

As the corresponding ascorbic acid analysis for the optical test showed, the batches with DPNH contain at the end of the test the entire added ascorbic acid in the reduced form, while in the batches without DPNH the ascorbic acid content had dropped on the average by 10%. In order to illustrate this difference, which indicates a H2-transport function of ascorbic acid, we changed the test conditions so that ascorbic acid was oxidized to a great extent in

batches without DPNH (see table 2).

Table 2. H2-transfer from DPNH to ascorbic acid in the presence of mitochondria.

Test	Total ascorbic acid mg		Ascorbic acid mg		Dehydroascorbic acid mg	
	0 min.	20 min.	0 min.	20 min.	0 min.	20 min.
With mitochondria						
1	1.00	0.90	0.90	0.35!	0.10	0.55!
2 (+ DPNH)	1.00	1.00	1.00	1.00!	0.00	0.00!
Without mitochondr.						
3	0.70	0.60	0.45	0.25	0.25	0.35
4 (+ DPNH)	0.65	0.60	0.40	0.20	0.25	0.40

1 mitochondria (150 mg dry weight). 1 mg ascorbic acid ($5.7 \cdot 10^{-6}$ moles), cytochrome C (4.10^{-7} moles); 2 like 1 + DPNH ($1.2 \cdot 10^{-5}$ moles) 3; ascorbic acid ($5.7 \cdot 10^{-6}$ mole), cytochrome C (4.10^{-7} moles); 4 like 3 + DPNH ($1.2 \cdot 10^{-5}$ moles). The total volume of the finished test batch is 3 ml (Krebs-ringer-phosphate buffer pH 6.6). 0 min.: immediate determination; 20 min: determination after an incubation period of 20 min (t 37 deg.C).

In the system ascorbic acid-mitochondria-cytochrome C without DPNH, 10% of the ascorbic acid are immediately oxidized to dehydroascorbic acid after the addition of cytochrome C. After 20 minutes, 55% of the added ascorbic acid have passed over altogether into dehydroascorbic acid. (table 2, test 1). But if DPNH is contained in this sytem, the ascorbic acid content remains constant (table 2, test 2). In the pure buffer system without mitochondria, the batches with and without DPNH do not differ from each other. The ascorbic acid is partly oxidized immediately by the cytochrome to dehydroascorbic acid and beyond that (table 2, tests 3 and 4). The same investigations were carried out with dehydroascorbic acid. The addition of DPNH to the model system dehydroascorbic acid -mitrochondria-cytochrome C does not effect a reduction of the dehydroascorbic acid to ascorbic acid. These findings are in good agreement with the results of the optical test.

Fig. 2. Effect of D-isoascorbic acid and of different L-ascorbic acid concentrations on the DPNH dehydration . 10 ml Krebs-Ringer-phosphate buffer, ATP 1 mu mole, versene 16 mu moles. Mitochondria of 100 mg adrenal glands. DPNH 1 mu mole. Ordinate; extinction at 340 m/mu; abscissa; time.

3. On the specificity of the ascorbic acid effect.

The acceleration of the DPNH dehydration depends, as shown in fig. 2, on the amount of ascorbic acid added. With mitochondria alone, 33% DPNH have been converted to DPN after 30 minutes. When 5 mu moles L-ascorbic acid are added, 65% DPNH are converted to DPN, and with the addition of 25 mu mole 93% are converted. An even higher concentration shows no further increase of the effect. In order to achieve the same effect with D-isoascorbic acid (1) as with 5 mu moles L-ascorbic acid, we have to add 25 mu moles. The D-isoascorbic acid is in these tests thus about 1/5 as effective as L-ascorbic acid.

Corresponding tests with TPNH (obtained by reacting TPN with sodium dithionite) led to the following result: TPNH is oxidized by adrenal gland-mitochondria, just like DPNH. The TPNH dehydration is only accelerated by L-ascorbic acid, however, in the presence of DPN. Under the above mentioned test conditions, 35% TPNH have been converted to TPN by mitochondria after 30 minutes; after the addition of 25 mu moles ascorbic acid and DPN the TPNH decrease was 90%.

Mitochondria, which were heated for 3 minutes to 60 deg. C, can no longer oxidize DPNH, not even after the addition of ascorbic acid.

4. Measuring the 02-consumption.

Only after the optimum test conditions for the ascorbic acid effect had been determined by means of the optical test could we demonstrate the effect of ascorbic acid in the Warburg apparatus too. In analogy to the optical test, DPNH was used as a substrate and the oxidation of DPNH by mitochondria with and without the addition of ascorbic acid was measured by the consumption of 02. At the end of the test samples were taken from the Warburg vessels which contain ascorbic acid, and the ascorbic acid content was determined. The test result is shown in fig. 3.

Fig. 3. The effect of ascorbic acid on the substrate oxidation by mitochondria. Warburg vessel 3 ml; 0.2 ml 10% KOH in the inner vessel. Mitochondria corresponding to 40 mg NN fresh weight .8 mu moles versene, 1 mu mole ATP. After a tempering time of 10 minutes added from the side arm: 8 mu moles DPNH, 5 mu moles ascorbic acid, 37 deg.C. Oscillation 100/min, pH 6.6 - 6.7. Ordinate: oxygen consumption. Abscissa: time.

The mitochondria (curve 1) have practically no endogenous breathing. DPNH is used up very little at the low mitrochondria concentration. (curve 2). The oxidation of ascorbic acid in the mitrochondria suspension is very low in the presence of versene (curve 3). In the batch DPNH + mitrochondria + ascorbic acid we found a very high oxygen consumption (curve 4).

The analysis of the ascorbic acid showed that 50% more ascorbic acid is retained in the batches with mitochondria + ascorbic acid + DPNH than in the batches with mitochondria + ascorbic acid alone. Under the test conditions given the Warburg apparatus, ascorbic acid is partly oxidized. In contrast to our batches in the optical test, we found a much higher oxygen saturation in the Warburg vessels by the larger surface and the oscillation. The resulting oxidation of the ascorbic acid cannot be completely suppressed by ethylene diamine tetraacetic acid.

In concentrated tissue homogenates the autoxidation of ascorbic acid is inhibited under the same conditions in the first 90 minutes (37 deg.C, oscillation of 100/min)[2].

This initial delay of the ascorbic acid oxidation is not observed in mitochondria suspensions.

If the oxidation of ascorbic acid extended in tissue-containing batches only up to dehydroascorbic acid, we would calculate from the corresponding ascorbic acid analyses the portion of the total 02-consumption due to this transformation. But the oxidation of ascorbic acid goes beyond dehydroascorbic acid

after about 60 minutes, forming products which have not yet been determined exactly by analysis (pentosene?, petrosene?). In this case reliable calculations of the oxygen consumption from the ascorbic acid loss are not possible. In fig. 3, curve 4, the increased additional breathing is far above what could be expected corresponding to the vanished ascorbic acid, even assuming oxidation up to a "tetrose".

As it was found in the optical determination of the DPNH content, the DPNH had been dehydrated completely to DPN in test batch, 4, while in test batch 2 without ascorbic acid 90% DPNH were still present at the same time. We are thus actually dealing here with an activation of the DPNH oxidation by ascorbic acid.

III. On the mechanism action of ascorbic acid.

According to observations by Kern (8), the electrochemical oxidation of ascorbic acid to dehydroascorbic acid takes place in two stages, with an unstable radical-type intermediate product being formed. We assumed that such an intermediate product also appears in the biological oxidation of ascorbic acid, particularly since the hydrogen- or electron transport in the oxidation processes in the cell can take place over radicals as intermediate stages according to experimentally found concepts by R. Kuhn (13, 14), and radical-type monodehydro-compounds could be demonstrated for the flavine system (summary in (15)).

Since ascorbic acid absorbs in the ultraviolet region (265 and 245 m mu resp.) and UV light can accelerate the formation of radicals we investigated if it is possible to enhance the formation of effective radicals from ascorbic acid by careful irradiation of our test batches with UV light. Similar tests regarding the influence of UV light on the ascorbic acid effect in the corticosteroid biosynthesis have already been discussed (11).

The measuring solution of the optical test is prepared as described above, and carried out in a double test series, but in 100 ml Erlenmeyer flasks of quartz glass. Both series are kept in the waterbath at the same temperature.

One series is irradiated with polychromatic UV light from a distance of 30 cm. Before each measurement the batches are shaken and then poured into the cuvettes.

Fig. 4. Effect of ascorbic acid + UV light on the DPNH dehydration by adrenal gland mitochondria. 10 ml Krebs-Ringer phosphate buffer pH 6.6, versene 16 mu moles; 1. DPNH 1 mu mole + ascorbic acid 25 mu moles + UV light; 2. DPNH + mitochondria of 100 mg adrenal glands; 3. like 2 + UV light; 4. like 2 + ascorbic acid 5 mu moles; 5. like 4 + UV light; 6. like 2 + ascorbic acid 25 mu moles; 7. like 6 + UV light. Arrow: addition of alcohol + alcohol dehydrogenase. Ordinate: extinction at 340 m mu. Abscissa time.

The effect of the UV light can be seen from fig. 4. UV light has no effect on the DPNH dehydration by adrenal gland mitochrondria with a low ascorbic acid content (fig. 4, curve 3). In test batches to which ascorbic acid was added, an activating effect of the UV light can be observed (fig. 4, curves 5 and 7). Even after addition of cytochrome C, UV light is only effective in the presence of ascorbic acid.

In order to check if the effect of the UV light is caused by the formation of ozone or peroxide, we also carried out the tests in the presence of katalase. They show the same course. The ascorbic acid content remains constant in all tests from the beginning to the end of the measurement (30 minutes). Dehydroascorbic acid is not formed. During brief irradiatin with UV light, the DPNH is not destroyed, as on prolonged irradiation (20), where the DPN formed is completely rehydrated at the end of the test (fig. 4).

IV Discussion

Our working hypothesis that the ascorbic acid effect in the corticosteroid biosynthesis of the adrenal cortex is due to an activation of the cellular breathing is further substantiated by the findings shown here.

Ascorbic acid is contained in the structures responsible for the oxidiative metabolic processes of the cell (mitochondia) in a concentration which is also assumed for other coenzymes of cellular breathing. The accelerating effect of ascorbic acid on the DPNH oxidation could be demonstrated both by means of the optical test and by the oxygen consumption in the Warburg apparatus. The ascorbic acid must thus be capable of transporting the hydrogen of DPNH, the hydrogen acceptor proper, the "effective ascorbic acid form" being formed intermediately. That the hydrogen of DPNH can actually be taken over by such an "intermediate product" (monodehydroascorbic acid) in the presence of mitochondria has been shown by our ascorbic acid analyses.

The enzyme system contained in the mitochondria has only a relative specificity for L-ascorbic acid, because D-iso-ascorbic acid is likewise effective in our investigations in the optical test, though only 1/5 of the L-ascorbic acid[3]. It seems as if the enzyme systems a specific for L-ascorbic acid also respond to isomers of ascorbic acid, but to a lesser extent than to L-ascorbic acid itself. The question of the specificity can only be answered reliably, however, when it is possible to isolate the enzyme whose effect has been proven (DPN-ascorbic acid-transhydrase).

Beevers (1), in his investigations of the effect of ascorbic acid on the DPNH dehydration in the presence of vegetable enzyme systems, could not replace DPNH by TPNH. In our system ascorbic acid activates the TPNH dehydration only in the presence of DPN. Since Sweat (21) has found a TPN-DPN transhydrate in adrenal glandmitochondria, the effect of ascorbic acid on the DPNH dehydration in the tests with adrenal gland mitrochondria can be readily accounted for.

The observation that UV light increases the ascorbic acid effect on the corticosteroid biosynthesis (11) and the DPNH dehydration leads directly to the question whether the activating influence of ascorbic acid is due to intermediately formed peroxide, particularly since H202 is formed in the autox-

idation of ascorbic acid, as known. But it can be argued as follows: 1. Peroxide can only be formed if ascorbic acid is oxidized. The ascorbic acid would have to be oxidized in these test batches at least up to dehydroascorbic acid. This is not the case, however, as our ascorbic acid analses have shown. 2. The much weaker effect of D-isoascorbic acid is not in agreement with such a non-specific effect of ascorbic acid. 3. We have shown that there is a saturation concentration for ascorbic acid. 4. The effect of ascorbic acid is not influenced by katalase.

It still has to be investigated whether the determined ascorbic acid effect on the DPNH oxidation is linked with the formation of high-energy phosphates, and whether the effect of ascorbic acid observed with adrenal gland mitochondria is of general significance. Furthermore, the question has to be clarified at which point the ascorbic acid intervenes in the electron transport.

Summary

1. Mitochondria of the adrenal glands of pigs still contain 30 mg ascorbic acid per 100 g mitochondria (dry weight) after repeated washings.

2. Ascorbic acid accelerates the DPNH dehydration caused by mitochondria.

3. Ascorbic acid is retained in the reduced form. It can take over the hydrogen from DPNH in the presence of mitochondria. Dehydroascorbic acid can not do this. It is also otherwise ineffective.

4. The effect of L-ascorbic acid in the above described system is relatively specific.

5. The importance of ascorbic acid for the breathing can also be demonstrated by the oxyten consumption in the Warburg apparatus.

6. The effective form of the ascorbic acid is presumably a radical-type "mono-dehydroascorbic acid".

We are indebted to Deutsche Forschungsgemeinschaft, which made these studies possible by their general support.

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Footnotes

1. We are indebted to Prof.Dr. Reichstein, Dr. L.W. Mapson and to Hoffman-La Roche for making D-isoascorbic acid available to us.

2. Unpublished tests by E. Kraushaar-Baldauf.

3. In addition Isherwood and coworkers (5) have shown that rats can form both L-ascorbic acid and D-isoascorbic acid from the corresponding initial products.

Translated by Carl Demrick Associates, Inc./IE/db

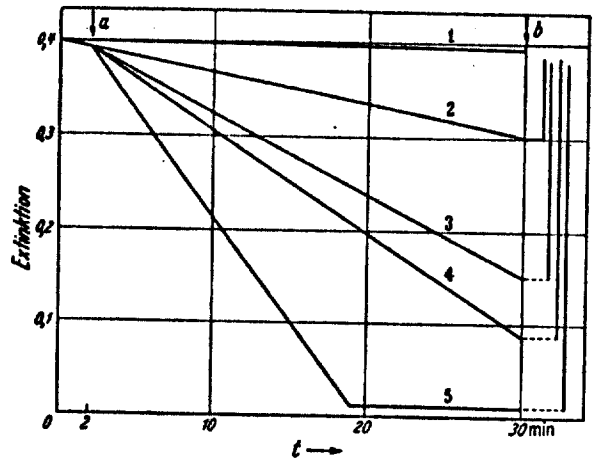

Figure 1

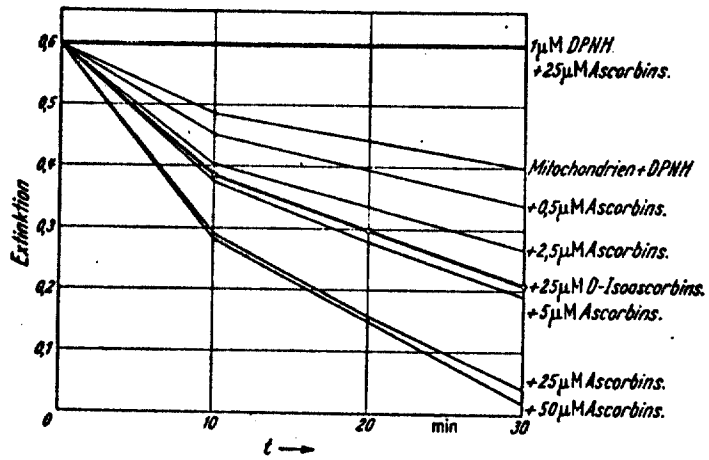

Figure 2

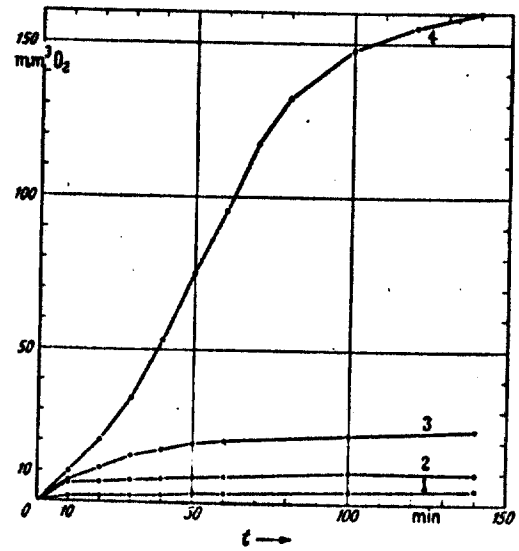

Figure 3

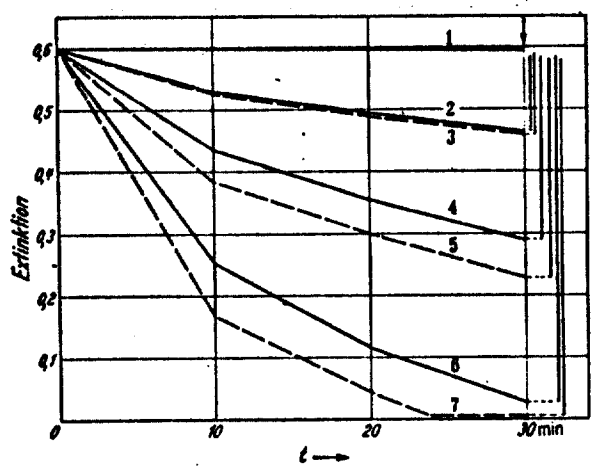

Figure 4

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Zum Wirkungsmechanismus der Ascorbinsäure

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Mit 4 Textabbildungen

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In Übereinstimmung mit WETTSTEIN u. Mitarb. [7] haben wir bei zahlreichen Untersuchungen den aktivierenden Einfluß der Ascorbinsäure auf die gesamte Corticosteroidbiosynthese und auf die 11 β-Hydroxylierung in vitro beobachtet [17]. Aus diesen Untersuchungen ergab sich die Frage nach dem Mechanismus der Ascorbinsäurewirkung speziell in der Nebenniere und darüber hinaus im tierischen Gewebe überhaupt.

Wir haben vermutet, daß die Ascorbinsäurewirkung auf eine Aktivierung des Wasserstoff- bzw. Elektronentransportes in der Zelle zurückzuführen ist. Ausgehend von dieser Hypothese wurde von uns mit Hilfe des optischen Testes der Verlauf der DPNH-Dehydrierung[1] in Gegenwart von Nebennierenmitochondrien nach Zusatz von Ascorbinsäure quantitativ verfolgt. Wie wir schon berichtet haben [12], wirkt Ascorbinsäure in diesem System beschleunigend auf die DPNH-Dehydrierung. Ähnliche Beobachtungen haben eine Reihe anderer Autoren bei Untersuchungen mit pflanzlichen Enzymsystemen gemacht [1, 9, 16].

Da Dehydroascorbinsäure bei denselben Versuchen keine Wirkung zeigt, ergab sich die weitere Frage, wie der Wasserstofftransport durch Ascorbinsäure vermittelt werden kann.

Wie wir in Übereinstimmung mit anderen Autoren [1, 9] vermuten, ist der eigentliche Wasserstoffacceptor eine radikalartige „Monodehydroascorbinsäure". Da der direkte Nachweis eines solchen Radikals bisher nicht möglich war, versuchten wir, unsere Annahme auf indirektem Wege zu stützen [10].

Auf Grund der besprochenen Befunde war zu prüfen, ob in den Mitochondrien selbst Ascorbinsäure enthalten ist. Um weiteren Aufschluß über den Mechanismus der Ascorbinsäurewirkung zu gewinnen, haben wir die Untersuchungen über die Wirkung der Ascorbinsäure auf die DPNH-Dehydrierung mit dem optischen Test weitergeführt. Die Bedeutung der Ascorbinsäure für die Zellatmung versuchten wir außerdem

[1] In der vorliegenden Arbeit werden folgende Abkürzungen verwandt: DPN = Diphosphopyridinnucleotid, DPNH = Dihydro-diphosphopyridinnucleotid, TPN = Triphosphopyridinnucleotid, TPNH = Dihydro-triphosphopyridinnucleotid.

durch Messung des Sauerstoffverbrauches in der WARBURG-Apparatur zu zeigen. Ferner wurde untersucht, inwieweit Ascorbinsäure Wasserstoff vom DPNH übernehmen kann.

I. Bestimmung des Ascorbinsäuregehaltes von Nebennierenmitochondrien

Wenn Ascorbinsäure eine Funktion im Wasserstofftransport der Zellatmung hat, dann müßte sie wie die anderen Glieder der Atmungskette, z. B. Cytochrom C, DPN usw., in den Mitochondrien enthalten sein. Histochemische Befunde [2] weisen zwar darauf hin, daß Ascorbinsäure in Mitochondrien vorkommt, doch lassen sich mit diesen Untersuchungsmethoden keine genauen Aussagen über Lokalisation und Menge der Ascorbinsäure in Mitochondrien machen [3]. Es wurden daher die von uns bei allen Experimenten verwendeten Schweinenebennieren-Mitochondrien papierchromatographisch [18] auf ihren Ascorbinsäuregehalt untersucht. Um zu sichern, daß es sich nicht um Ascorbinsäure handelt, die beim Arbeitsgang mitgeschleppt wurden, haben wir die Mitochondrien 1—3 mal mit der 10fachen Menge isotoner Saccharoselösung gewaschen.

Tabelle 1. *Ascorbinsäuregehalt in Nebennierenmitochondrien*

Mitochondrien von 1 g frischen Nebennieren	Milligramm AS i. Mitochondrien von 1 g frischen Nebennieren	Milligramm AS bezogen auf 1 g Mitochondrien Trockengewicht
1 × gewaschen	0,030	0,60
2 × gewaschen	0,015	0,30
3 × gewaschen	0,013	0,26

Der Ascorbinsäuregehalt von Schweinenebennieren beträgt durchschnittlich 200 mg-% [6]. Davon ist, wie aus der Tab. 1 hervorgeht, etwa 1% in den Mitochondrien lokalisiert und offenbar relativ fest gebunden.

II. Untersuchungen über die Wirkung der Ascorbinsäure auf den Wasserstofftransport

Wie schon berichtet [12] haben wir bei früheren Untersuchungen mit dem optischen Test gezeigt, daß DPNH durch Nebennierenmitochondrien in Gegenwart von Ascorbinsäure und Cytochrom C + Ascorbinsäure schneller oxydiert wird, als ohne die genannten Aktivatoren. Zu beweisen war aber noch, daß DPNH dabei quantitativ in DPN übergeht und außerdem Ascorbinsäure den Wasserstoff vom DPNH übernimmt. Zu diesem Zweck wurde am Ende des optischen Testes entstandenes DPN enzymatisch zu DPNH reduziert und Ascorbinsäure papierchromatographisch bestimmt. Um einen Hinweis zu erhalten, ob es sich bei der Ascorbinsäurewirkung um einen enzymatischen Prozeß handelt, wurde versucht, eine Sättigungskonzentration der Ascorbinsäure zu bestimmen.

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Zur Klärung der Frage, inwieweit der beobachtete Effekt für L-Ascorbinsäure spezifisch ist, wurden entsprechende Ansätze mit Isoascorbinsäure (= D-Araboascorbinsäure) im optischen Test gemessen.

1. *DPNH-Dehydrierung*

a) Experimentelles. Schweinenebennieren werden, wie früher beschrieben [12], nach der Methode von HOGEBOOM, SCHNEIDER u. PALLADE [4] und SCHNEIDER [19] durch fraktioniertes Zentrifugieren in 0,88 m Saccharoselösung gewonnen und mit 0,25 m Saccharose gewaschen. Die Saccharoselösungen enthalten Versen ($1 \cdot 10^{-3}$ m).

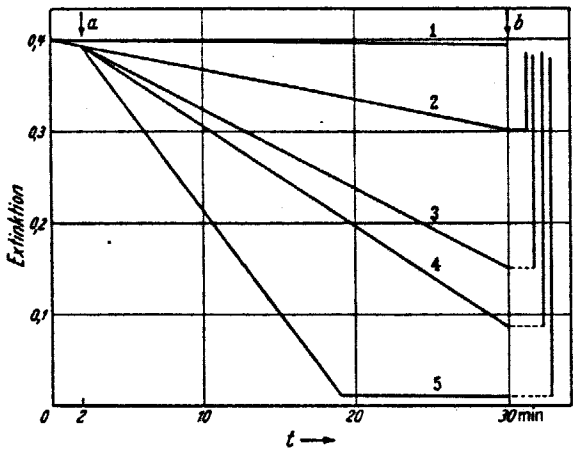

Abb. 1. Die Wirkung von Ascorbinsäure und Cytochrom C auf die DPNH-Dehydrierung durch Nebennierenmitochondrien. In 10 ml KREBS-Ringer-Phosphat-Puffer: 1 μMol ATP, 16 μMol Versen. 1. DPNH 0,7 μMol + Ascorbinsäure 10 μMol + Cytochrom C 0,01 μMol; 2. DPNH + Mitochondrien von 100 mg Nebennieren; 3. wie 2 + Ascorbinsäure 10 μMol; 4. wie 2 + Cytochrom; 5. wie 3 + Cytochrom + Ascorbinsäure. ↓ a Zugabe von Ascorbinsäure und Cytochrom. ↓ b Zugabe von Alkohol und Alkoholdehydrogenase. Ordinate: Extinktion bei 340 mμ; Abszisse: Zeit

Mitochondrien von 20 g Nebennieren werden in KREBS-Ringer-Phosphat- oder auch SÖRENSEN-Puffer von p_H 6,8 aufgenommen. Die so gewonnene Suspension wird auf 20 ml aufgefüllt. Der p_H-Wert dieser Stammsuspension beträgt dann p_H 6,6—6,7. Der Mitochondriengehalt von 1 ml Stammsuspension entspricht 1 g Nebennieren (Frischgewicht). Die Meßlösung für den optischen Test enthält jeweils die Mitochondrien von 0,04—0,1 g Nebennieren, Versen ($1,6 \cdot 10^{-3}$ m) und ATP ($0,5 \cdot 10^{-4}$ m) und die Zusätze in insgesamt 10 ml Puffer. Alle Lösungen, Reagentien und Zusätze werden bis zum Beginn der Messung eiskalt gehalten.

Nach Bereitung der Ansätze werden diese gut durchgeschüttelt und der Extinktionsverlauf bei 37° C im Zeiß-Spektralphotometer in 1 cm-Cuvetten bei 340 mμ verfolgt. Der Abfall der Eigenextinktion der Mitochondrien muß zu jedem Zeitpunkt bestimmt werden und wird bei der graphischen Darstellung eliminiert.

b) Versuchsergebnisse. Abb. 1 zeigt den typischen Verlauf eines optischen Testes. Die reinen Substanzen DPNH-Cytochrom-Ascorbinsäure reagieren nicht miteinander (Kurve 1). Nebennierenmitochondrien bewirken in der verwendeten Konzentration nur eine geringe Dehydrierung

von DPNH (Kurve 2), die durch Zusatz von Ascorbinsäure beschleunigt wird (Kurve 3). Zugefügtes Cytochrom C wirkt ebenfalls aktivierend auf die Oxydation von DPNH (Kurve 4). Der Effekt kann durch Ascorbinsäure weiter erhöht werden (Kurve 5). Hierbei ist das vorgelegte DPNH nach etwa 20 min vollständig verbraucht. Bei einer anschließenden enzymatischen Reduktion erhält man in allen Ansätzen die Extinktion die dem eingesetzten DPNH entspricht. Daraus geht hervor, daß das DPNH nur dehydriert und nicht an anderer Stelle verändert wird.

2. *Wasserstofftransportfunktion der Ascorbinsäure*

Wie die entsprechenden Ascorbinsäureanalysen zum optischen Test ergaben, enthalten die Ansätze mit DPNH am Ende des Versuches die gesamte zugesetzte Ascorbinsäure in der reduzierten Form, während in den Ansätzen ohne DPNH der Ascorbinsäuregehalt durchschnittlich um 10% gesunken war. Um diesen Unterschied, der für eine H_2-Transportfunktion der Ascorbinsäure spricht, deutlich zu machen, wurden die Versuchsbedingungen so geändert, daß Ascorbinsäure in Ansätzen ohne DPNH weitgehend oxydiert wurde (vgl. Tab. 2).

Tabelle 2. *H_2-Übertragung vom DPNH auf Ascorbinsäure in Gegenwart von Mitochondrien*

Versuch	Gesamtascorbinsäure mg		Ascorbinsäure mg		Dehydroascorbinsäure mg	
	0 min	20 min	0 min	20 min	0 min	20 min
Mit Mitochondrien						
1	1,00	0,90	0,90	0,35!	0,10	0,55!
2 (+ DPNH)	1,00	1,00	1,00	1,00!	0,00	0,00!
Ohne Mitochondrien						
3	0,70	0,60	0,45	0,25	0,25	0,35
4 (+ DPNH)	0,65	0,60	0,40	0,20	0,25	0,40

1 Mitochondrien (150 mg Trockengewicht), 1 mg Ascorbinsäure ($5,7 \cdot 10^{-6}$ Mol), Cytochrom C ($4 \cdot 10^{-7}$ Mol); 2 wie 1 + DPNH ($1,2 \cdot 10^{-5}$ Mol); 3 Ascorbinsäure ($5,7 \cdot 10^{-6}$ Mol), Cytochrom C ($4 \cdot 10^{-7}$ Mol); 4 wie 3 + DPNH ($1,2 \cdot 10^{-5}$ Mol). Das Gesamtvolumen des fertigen Versuchsansatzes beträgt 3 ml (KREBS-RINGER-Phosphat-Puffer p_H 6,6). 0 min: Sofortbestimmung; 20 min: Bestimmung nach einer Bebrütungszeit von 20 min (*t* 37° C).

In dem System Ascorbinsäure-Mitochondrien-Cytochrom C *ohne* DPNH werden sofort nach Zugabe von Cytochrom C 10% der Ascorbinsäure zu Dehydroascorbinsäure oxydiert. Nach 20 min sind insgesamt 55% der zugesetzten Ascorbinsäure in Dehydroascorbinsäure übergegangen (Tab. 2, Versuch 1). Befindet sich in diesem System aber DPNH, so bleibt der Ascorbinsäuregehalt konstant (Tab. 2, Vers. 2). Im reinen Puffersystem ohne Mitochondrien unterscheiden sich die Ansätze mit

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und ohne DPNH nicht voneinander. Die Ascorbinsäure wird zum Teil sofort durch das Cytochrom zu Dehydroascorbinsäure und darüber hinaus oxydiert (Tab. 2, Vers. 3 u. 4). Die gleichen Untersuchungen haben wir mit Dehydroascorbinsäure durchgeführt. DPNH-Zusatz zu dem Modellsystem Dehydroascorbinsäure-Mitochondrien-Cytochrom C bewirkt keine Reduktion der Dehydroascorbinsäure zu Ascorbinsäure. Diese Befunde stimmen mit den Ergebnissen des optischen Testes gut überein.

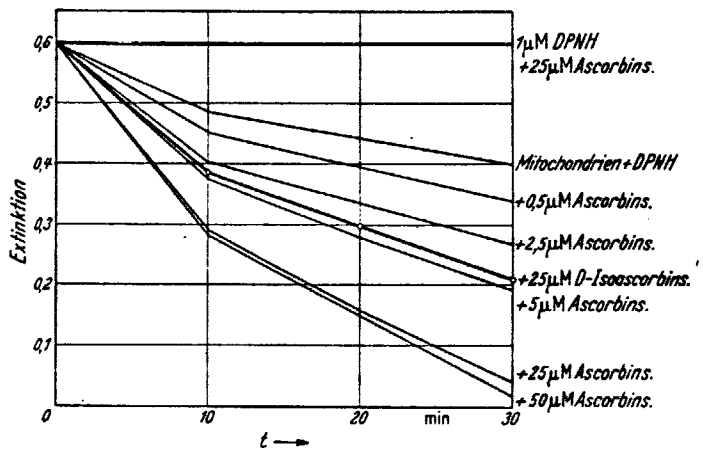

Abb. 2. Die Wirkung von D-Isoascorbinsäure und verschiedenen L-Ascorbinsäurekonzentrationen auf die DPNH-Dehydrierung. 10 ml KREBS-Ringer-Phosphat-Puffer, ATP 1 μMol, Versen 16 μMol. Mitochondrien von 100 mg Nebennieren. DPNH 1 μMol. Ordinate: Extinktion bei 340 mμ; Abszisse: Zeit.

3. *Zur Spezifität der Ascorbinsäurewirkung*

Die Beschleunigung der DPNH-Dehydrierung ist, wie Abb. 2 zeigt, abhängig von der zugesetzten Ascorbinsäuremenge. Mit Mitochondrien allein sind nach 30 min 33% DPNH in DPN umgewandelt. Bei Zugabe von 5 μMol L-Ascorbinsäure sind 65%, bei Zugabe von 25 μMol 93% DPNH zu DPN oxydiert. Eine noch höhere Konzentration zeigt keine weitere Wirkungssteigerung. Um mit D-Isoascorbinsäure[1] die gleiche Wirkung wie mit 5 μMol L-Ascorbinsäure zu erreichen, muß man 25 μMol zusetzen. Die D-Isoascorbinsäure ist bei diesen Versuchen also etwa 1/5 so wirksam wie L-Ascorbinsäure.

Entsprechende Versuche mit TPNH (gewonnen durch Reduktion von TPN mit Natriumdithionit) führten zu folgendem Ergebnis: TPNH wird wird von Nebennierenmitochondrien ebenso oxydiert wie DPNH. Die

[1] Wir sind Herrn Prof. Dr. T. REICHSTEIN, Herrn Dr. L. W. MAPSON und der Firma Hoffmann-La Roche für die Überlassung von D-Isoascorbinsäure zu Dank verpflichtet.

TPNH-Dehydrierung wird aber nur in Gegenwart von DPN durch L-Ascorbinsäure beschleunigt. Unter den ebengenannten Versuchsbedingungen sind durch Mitochondrien nach 30 min 35% TPNH in TPN umgewandelt, nach Zusatz von 25 μMol Ascorbinsäure und DPN betrug die TPNH-Abnahme 90%.

Mitochondrien, die 3 min auf 60° C erwärmt wurden, vermögen DPNH nicht mehr zu oxydieren, auch nicht nach Zusatz von Ascorbinsäure.

4. *Messung des O_2-Verbrauches*

Erst nachdem wir mit Hilfe des optischen Testes optimale Versuchsbedingungen für die Ascorbinsäurewirkung festgelegt hatten, konnten wir die Wirkung der Ascorbinsäure auch in der WARBURG-Apparatur nachweisen. Analog zum optischen Test wurde DPNH als Substrat eingesetzt und die Oxydation des DPNH durch Mitochondrien mit und ohne Zusatz von Ascorbinsäure am O_2-Verbrauch gemessen. Aus den WARBURG-Gläschen, die Ascorbinsäure enthielten, wurden am Ende des Versuches Proben entnommen und der Ascorbinsäuregehalt bestimmt. Das Versuchsergebnis ist in Abb. 3 dargestellt.

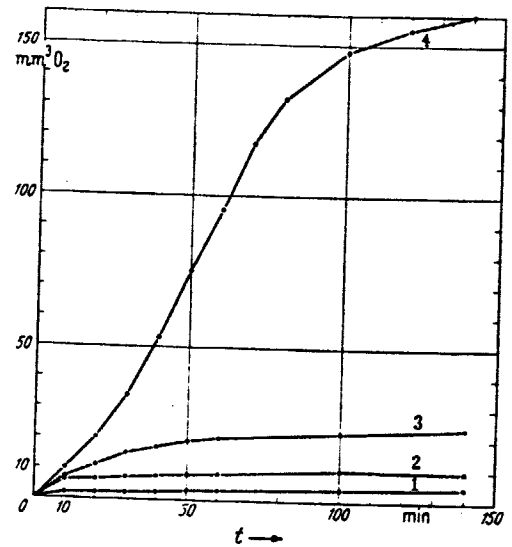

Abb. 3. Die Ascorbinsäurewirkung auf die Substratoxydation durch Mitochondrien. WARBURG-Gefäß 3 ml: 0,2 ml 10% KOH im Innengefäß. Mitochondrien entsprechend 40 mg NN-Frischgewicht. 8 μMol Versen, 1 μMol ATP. Nach der Temperierzeit von 10 min aus dem Seitenarm zugegeben: 8 μMol DPNH, 5 μMol Ascorbinsäure. 37° C, Oscillation 100/min, p_H 6,6—6,7. Ordinate: Sauerstoffverbrauch; Abszisse: Zeit

Die Mitochondrien (Kurve 1) haben fast keine endogene Atmung. DPNH wird bei der geringen Mitochondrienkonzentration nur sehr wenig veratmet (Kurve 2). Die Oxydation der Ascorbinsäure in der Mitochondriensuspension ist in Gegenwart von Versen nur sehr gering (Kurve 3). In dem Ansatz DPNH + Mitochondrien + Ascorbinsäure zeigt sich ein sehr starker Sauerstoffverbrauch (Kurve 4).

Die Ascorbinsäureanalysen ergaben, daß in den Ansätzen mit Mitochondrien + Ascorbinsäure + DPNH 50% mehr Ascorbinsäure erhalten bleibt, als in den Ansätzen mit Mitochondrien + Ascorbinsäure allein. Bei den in der WARBURG-Apparatur gegebenen Versuchsbedingungen

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wird Ascorbinsäure zum Teil oxydiert. Im Gegensatz zu unseren Ansätzen beim optischen Test haben wir in dem WARBURG-Gläschen durch die größere Oberfläche und die Oscillation eine sehr viel höhere Sauerstoffsättigung. Die dadurch bedingte Oxydation der Ascorbinsäure läßt sich auch durch Äthylendiamintetraessigsäure nicht völlig unterdrücken.

In konzentrierten Gewebehomogenaten wird die Autoxydation der Ascorbinsäure unter denselben Versuchsbedingungen in den ersten 90 min gehemmt (37° C, Oscillation von 100/min)[1].

Diese anfängliche Verzögerung der Ascorbinsäureoxydation ist in Mitochondriensuspensionen nicht zu beobachten.

Wenn die Oxydation der Ascorbinsäure in gewebehaltigen Ansätzen nur bis zur Dehydroascorbinsäure verliefe, könnte man aus entsprechenden Ascorbinsäureanalysen den auf diese Umwandlung entfallenden Anteil des Gesamt-O_2-Verbrauches berechnen. Die Oxydation der Ascorbinsäure geht aber nach etwa 60 min über die Dehydroascorbinsäure hinaus zu Produkten, die bisher analytisch nicht genau genug erfaßt worden sind (Pentosen?, Tetrosen?). In diesem Fall lassen sich sichere Berechnungen des Sauerstoffverbrauchs aus dem Ascorbinsäureverlust nicht durchführen. In Abb. 3, Kurve 4 liegt die beobachtete Mehratmung jedoch weit über dem, was der verschwundenen Ascorbinsäure auch bei Annahme einer Oxydation bis zu einer „Tetrose" entsprechen könnte.

Wie sich aus der optischen Bestimmung des DPNH-Gehaltes ergab, war in dem Versuchsansatz 4 das DPNH völlig zu DPN dehydriert, während im Versuchsansatz 2 ohne Ascorbinsäure zum gleichen Zeitpunkt noch 90% DPNH vorhanden waren. Es handelt sich also wirklich um eine Aktivierung der DPNH-Oxydation durch Ascorbinsäure.

III. Zum Mechanismus der Ascorbinsäurewirkung

Nach Beobachtungen von KERN [8] verläuft die elektrochemische Oxydation von Ascorbinsäure zu Dehydroascorbinsäure in zwei Stufen, wobei ein instabiles, radikalartiges Zwischenprodukt entsteht. Wir haben, vermutet, daß bei der biologischen Ascorbinsäureoxydation ebenfalls ein solches Zwischenprodukt auftritt, zumal nach experimentell begründeten Vorstellungen von R. KUHN [13, 14] der Wasserstoff- bzw. Elektronentransport bei den Oxydationsvorgängen in der Zelle über Radikale als Zwischenstufen verlaufen kann und für das Flavinsystem radikalartige Monodehydroverbindungen nachgewiesen werden konnten (zusammenfassend b. [15]).

Da Ascorbinsäure im ultravioletten Bereich (265 mμ bzw. 245 mμ) absorbiert und UV-Licht die Radikalbildung zu beschleunigen vermag,

[1] Unveröffentlichte Versuche von E. KRAUSHAAR-BALDAUF.

haben wir untersucht, ob es möglich ist, durch vorsichtiges Bestrahlen unserer Versuchsansätze mit UV-Licht die Entstehung von wirksamen Radikalen aus der Ascorbinsäure zu fördern. Ähnliche Versuche über den Einfluß von UV-Licht auf die Ascorbinsäurewirkung bei der Corticosteroidbiosynthese wurden schon besprochen [11].

Die Meßlösung des optischen Testes wird wie oben beschrieben und in doppelter Versuchsreihe, jedoch in 100 ml-Erlenmeyer-Kolben aus

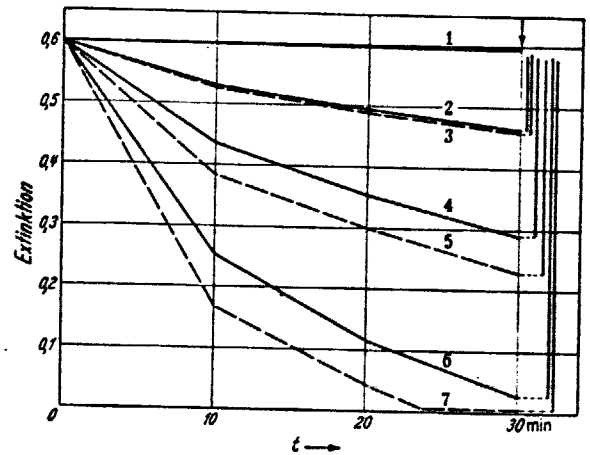

Abb. 4. Die Wirkung von Ascorbinsäure + UV-Licht auf die DPHN-Dehydrierung durch Nebennierenmitochondrien. 10 ml KREBS-Ringer-Phosphat-Puffer pH 6,6, Versen 16 μMol; 1. DPNH 1 μMol + Ascorbinsäure 25 μMol + UV-Licht; 2. DPNH + Mitochondrien von 100 mg Nebennieren; 3. wie 2 + UV-Licht; 4. wie 2 + Ascorbinsäure 5 μMol; 5. wie 4 + UV-Licht; 6. wie 2 + Ascorbinsäure 25 μMol; 7. wie 6 + UV-Licht. ↓ Zugabe von Alkohol + Alkoholdehydrogenase. Ordinate: Extinktion bei 340 mμ; Abszisse: Zeit

Quarzglas angesetzt. Im Wasserbad werden beide Reihen auf gleicher Temperatur gehalten. Eine Reihe wird mit polychromatischen UV-Licht aus 30 cm Entfernung bestrahlt. Vor jeder Messung werden die Ansätze umgeschüttelt, dann in die Cuvetten gegeben.

Die Wirkung des UV-Lichtes ist aus Abb. 4 ersichtlich. UV-Licht hat auf die DPNH-Dehydrierung durch ascorbinsäurearme Nebennierenmitochondrien keinen Einfluß (Abb. 4, Kurve 3). In Versuchsansätzen, denen Ascorbinsäure zugesetzt wurde, ist eine aktivierende Wirkung des UV-Lichtes zu beobachten (Abb. 4, Kurve 5 u. 7). Auch nach Zusatz von Cytochrom C ist UV-Licht nur in Gegenwart von Ascorbinsäure wirksam.

Um zu prüfen, ob die Wirkung des UV-Lichtes durch Ozon- bzw. Peroxydbildung zustande kommt, haben wir die Versuche auch in Gegenwart von Katalase durchgeführt. Sie zeigen den gleichen Verlauf. Der Ascorbinsäuregehalt bleibt auch in all diesen Versuchen von Beginn bis

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zum Ende der Messung (30 min) konstant. Dehydroascorbinsäure entsteht nicht. Bei der kurzfristigen Bestrahlung mit UV-Licht wird das DPNH nicht zerstört wie bei längerer Bestrahlung [20], das entstandene DPN läßt sich am Ende des Versuches völlig rückhydrieren (Abb. 4).

IV. Diskussion

Unsere Arbeitshypothese, daß die Ascorbinsäurewirkung bei der Corticosteroidbiosynthese der Nebennierenrinde auf eine Aktivierung der Zellatmung zurückzuführen ist, wird durch die dargelegten Befunde weiter gestützt.

Ascorbinsäure kommt in den für die oxydativen Stoffwechselprozesse der Zelle verantwortlichen Strukturen (den Mitochondrien) in einer Konzentration vor, wie sie auch für andere Coenzyme der Zellatmung angenommen wird. Die beschleunigende Wirkung der Ascorbinsäure auf die DPNH-Oxydation konnten wir sowohl mit Hilfe des optischen Testes als auch am Sauerstoffverbrauch in der Warburg-Apparatur nachweisen. Der Wasserstoff vom DPNH muß also von der Ascorbinsäure transportiert werden können, wobei der eigentliche Wasserstoffacceptor, die „Ascorbinsäurewirkform", intermediär gebildet wird. Daß der Wasserstoff vom DPNH in Gegenwart von Mitochondrien wirklich von einem solchen „Zwischenprodukt" (Monodehydroascorbinsäure?) übernommen werden kann, haben unsere Ascorbinsäureanalysen gezeigt.

Das in den Mitochondrien enthaltene Enzymsystem hat für L-Ascorbinsäure nur eine relative Spezifität, denn D-Isoascorbinsäure ist bei unseren Versuchen im optischen Test ebenfalls, wenn auch nur 1/5 so wirksam wie L-Ascorbinsäure. D-Isoascorbinsäure hat bekanntlich antiskorbutische Wirkung, jedoch weniger als L-Ascorbinsäure[1]. Es scheint so, als ob die für die L-Ascorbinsäure spezifischen Enzymsysteme auch auf Isomere der Ascorbinsäure ansprechen, jedoch in geringerem Maße als auf L-Ascorbinsäure selbst. Die Frage der Spezifität kann aber erst dann mit Sicherheit beantwortet werden, wenn es uns gelingt, das in seiner Wirkung nachgewiesene Enzym (DPN-Ascorbinsäuretranshydrase) zu isolieren.

Beevers [1] konnte bei seinen Untersuchungen über die Wirkung der Ascorbinsäure auf die DPNH-Dehydrierung in Gegenwart von pflanzlichen Enzymsystemen DPNH nicht durch TPNH ersetzen. In unserem System wirkt Ascorbinsäure nur in Gegenwart von DPN aktivierend auf die TPNH-Dehydrierung. Da Sweat [21] in Nebennierenmitochondrien eine TPN-DPN-Transhydrase nachgewiesen hat, läßt sich die Wirkung der Ascorbinsäure auf die TPNH-Dehydrierung bei den Versuchen mit Nebennierenmitochondrien zwanglos erklären.

Die Beobachtung, daß UV-Licht die Ascorbinsäurewirkung auf die Corticosteroidbiosynthese [11] und die DPNH-Dehydrierung noch erhöht, führt unmittelbar zu der Frage, ob nicht der aktivierende Einfluß von Ascorbinsäure auf intermediär gebildetes Peroxyd zurückzuführen ist, zumal bekanntlich bei der Autoxydation der Ascorbinsäure H_2O_2 entsteht. Dagegen ist folgendes einzuwenden: 1. Peroxyd kann nur dann entstehen, wenn Ascorbinsäure oxydiert wird. Die Ascorbinsäure müßte in diesen Versuchsansätzen mindestens bis zur Dehydroascorbinsäure oxydiert worden sein. Das ist aber, wie unsere Ascorbinsäureanalysen ergaben, nicht der Fall. 2. Die deutlich schwächere Wirkung von D-Isoascorbinsäure läßt sich nicht mit einer solch unspezifischen Wirkung der Ascorbinsäure in Einklang bringen. 3. Wir haben gezeigt, daß für die Ascorbinsäure eine Sättigungskonzentration existiert. 4. Die Wirkung der Ascorbinsäure wird durch Katalase nicht beeinflußt.

Es bleibt weiter zu untersuchen, ob mit der gefundenen Ascorbinsäurewirkung auf die DPNH-Oxydation die Entstehung energiereichen Phosphates verknüpft ist, und ob die mit Nebennierenmitochondrien beobachtete Wirkung der Ascorbinsäure allgemeinere Bedeutung hat. Ferner ist die Frage zu klären, an welcher Stelle die Ascorbinsäure in den Elektronentransport eingreift.

Zusammenfassung

1. Mitochondrien von Schweinenebennieren enthalten nach mehrmaligem Auswaschen noch 30 mg Ascorbinsäure pro 100 g Mitochondrien (Trockengewicht).

2. Ascorbinsäure beschleunigt die durch Mitochondrien bedingte DPNH-Dehydrierung.

3. Ascorbinsäure bleibt hierbei in der reduzierten Form erhalten. Sie kann in Gegenwart von Mitochondrien den Wasserstoff vom DPNH übernehmen. Dehydroascorbinsäure ist dazu nicht in der Lage; sie ist auch sonst unwirksam.

4. Die Wirkung der L-Ascorbinsäure in dem beschriebenen System ist relativ spezifisch.

5. Die Bedeutung der Ascorbinsäure für die Atmung läßt sich auch in der Warburg-Apparatur am Sauerstoffverbrauch zeigen.

6. Die Wirkform der Ascorbinsäure ist vermutlich eine radikalartige „Monodehydroascorbinsäure".

Wir sind der Deutschen Forschungsgemeinschaft sehr zu Dank verpflichtet, die uns durch ihre großzügige Unterstützung die Durchführung der Arbeiten ermöglichte.

[1] Außerdem haben Isherwood u. Mitarb. [5] gezeigt, daß Ratten sowohl L-Ascorbinsäure als auch D-Isoascorbinsäure aus den entsprechenden Vorprodukten bilden können.

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The Atypical Ascorbic Acid Oxidase in Fungus Spores—Its Inactivation by Isoascorbate and Its Specificity

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Introduction

In a previous paper (1) it was reported that spores of the fungus *Myrothecium verrucaria* have a surface-located enzyme which oxidizes ascorbic acid. Data were presented showing the enzyme to differ from previously described ascorbic acid oxidizing systems, the main differentiating characteristic being the resistance of the enzyme, *in vitro* or *in vivo*, to such poisons of heavy-metal systems as cyanide, azide, diethyl dithiocarbamate, phenylthiourea and 8-hydroxyquinoline. The present paper describes the high substrate specificity of the enzyme and additional studies based on the observation that the enzyme is strongly inhibited by isoascorbate. These characteristics further differentiate the enzyme from other ascorbate oxidizing systems.

Methods

The methods used were essentially those described previously (1). Spores of the fungus *Myrothecium verrucaria* were obtained from cultures grown on filter paper. Since it was found that toluene-treated spores behaved, in all matters studied, identically to spore extracts (unpublished), they were used in most of the experiments because of the relative ease of preparation or because their use enabled the "enzyme" to be treated with inhibitor and washed by centrifugation. Furthermore, since the spores are killed by this treatment, the complications of metabolic reactions which would interfere with the manometric assay of enzymic activity are avoided. The toluene-treated spores were prepared by shaking a suspension of spores with toluene for 1 hr. at 30°C., centrifuging, and washing several times. This treatment causes no change in the ascorbic acid oxidase activity of the spores.

Ascorbic acid oxidase activity was assayed by conventional Warburg manometric techniques to measure oxygen uptake during the oxidation of ascorbic acid at pH 6.3 in 0.05 *M* KH_2PO_4-K_2HPO_4 buffer. Unless stated to the contrary, the

vessels contained 3 mg. ascorbate and a total fluid volume of 1.5 ml. Glutathione was not added as in the previous communication except where indicated. L-ascorbic acid (AA) and D-isoascorbic acid (isoAA) were obtained from Eastman Kodak Company, and D-glucoascorbic acid from Delta Chemical Company. Ascorbic acid oxidase prepared from squash was obtained from Delta Chemical Company, and was diluted with 0.5% gelatin before use (2).

Results

1. *Substrate Specificity*

Comparison of the oxidation of ascorbic acid and several of its analogs by a spore extract and by a preparation of ascorbic acid oxidase from squash shows a marked difference between the action of the two preparations (Table I). While the enzyme from squash readily oxidizes all three of the substrates tested (L-ascorbic, D-isoascorbic, and D-glucoascorbic acids), the enzyme from *M. verrucaria* spores oxidizes only the L-ascorbic acid. With mixtures of the substrates, isoascorbate completely blocks oxidation of ascorbate by the spore extract, whereas glucoascorbate has no such inhibitory action.

TABLE I

Comparison of Substrate Specificity of Ascorbic Acid Oxidases

Substrate[a]	μl. O_2 absorbed @ 15 min. Spore extract	"Squash" oxidase[b]	No enzyme
L-Ascorbic acid	89	137	0
D-Isoascorbic acid	4	140	2
D-Glucoascorbic acid	0	99	0
Ascorbic + isoascorbic	3	141	—
Ascorbic + glucoascorbic	104	147	—
Glucoascorbic + isoascorbic	0	145	—

[a] Contained 3 mg./vessel; where two substrates, 3 mg. of each/vessel.
[b] Squash oxidase obtained from Delta Chemical Company, diluted with 0.5% gelatin.

2. *Inhibition by Isoascorbate*

Experiments were carried out to study the inhibitory action of isoascorbate in greater detail. In the course of this work duplication of results was not always satisfactory when low concentrations of isoascorbate were employed. This was found to be due, apparently, to autoxidation of the isoascorbate, the oxidation products being ineffective as inhibitors. For this reason, freshly prepared solutions of inhibitor were

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always used, and in some experiments (wherever indicated) the isoascorbate was dissolved in buffer containing glutathione (30 μg./ml.) to prevent or retard this oxidation.

The close structural similarity between ascorbate and isoascorbate suggests that the latter acts as a competitive inhibitor. Tests to establish the nature of the inhibition showed the degree of inhibition to be essentially independent of the substrate concentration (Table II). This would seem to indicate a noncompetitive relationship. It is perhaps significant, however, that the reaction rate is not greatly influenced by substrate concentrations over the range employed here. Unfortunately the techniques used would not permit measurements at lower substrate concentrations.

TABLE II

Effect of Isoascorbic Concentration on Inhibition of Spore Extracts at Different Substrate Concentrations

Spore extract incubated 1 hr. with isoascorbate before tipping in ascorbate. IsoAA = isoascorbic acid; AA = ascorbic acid; quantities given as per vessel containing 1.5 ml. of solution.

	μl. O_2 @ 10 min.			Inhibition, per cent		
iso AA	1 mg. AA	4 mg. AA	16 mg. AA	1 mg. AA	4 mg. AA	16 mg. AA
0μg.	56	61	70	0	0	0
5	46	46	61	18	25	13
10	34	26	30	39	57	57
15	13	9	15	77	85	79
20	4	6	—	93	90	—
25	2.5	5	10	95	92	86

The structural relationships of sugars to ascorbic acid suggested that they might have inhibitory effects on the enzyme. Tests using D(−)-arabinose, L(+)-arabinose, D(+)-galactose, D(+)-mannose, D(+)-xylose, L(−)-sorbose, D(−)-ribose, and D(+)-glucose showed no effects on the oxidation of ascorbate by spore extracts (3 mg. ascorbate, 10 mg. sugar/vessel).

At low concentrations of isoascorbate the inhibition is not instantaneous. Results of a typical experiment are shown in Fig. 1, in which 0.02 mg. isoascorbate was added to spore extract from one side arm at varying times up to 64 min., at which time ascorbate (6 mg.) was tipped into the vessel from a second side arm. When isoascorbate and ascorbate are tipped in simultaneously (see 0 min. curve) significant inhibition is noted only after 5 or 10 min., the extent of inhibition increasing with

time. When isoascorbate is added prior to the ascorbate, the initial inhibition is a function of the time of previous exposure. Apparently the inhibition in the presence of ascorbate is less than in its absence.

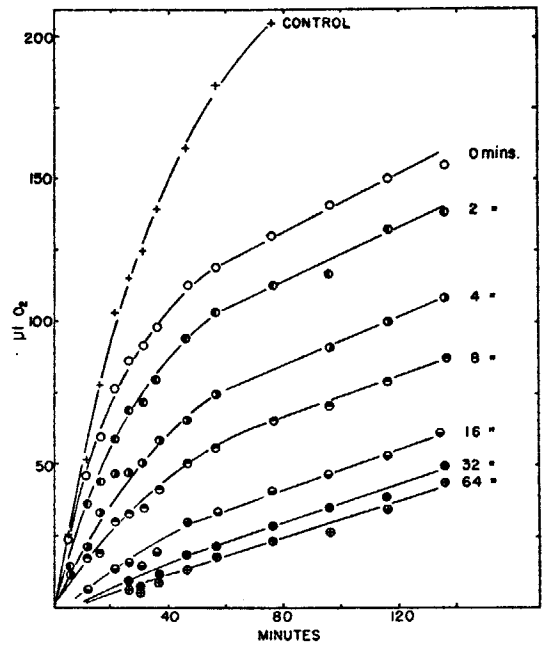

FIG. 1. Effect of preincubation with isoascorbate on inactivation of enzyme in spore extract. Twenty micrograms isoascorbate in 0.5 ml. buffer tipped into 1 ml. of extract at indicated time before adding 6 mg. ascorbate in 0.5 ml. buffer from second side arm.

Thus greater inhibition is noted where the preparation was preincubated with isoascorbate than where exposure to both substrate and inhibitor was for the same period of time; for example, at 16 min. the slope of the curve for 0 min. preincubation is much greater than the initial slope of the 16-min. preincubation curve (Fig. 1). This evidence is suggestive

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of there actually being some competition between substrate and inhibitor, contrary to the data of Table II.

Under the conditions of these experiments the initial rate of oxidation of ascorbate is proportional to the enzyme concentration (unpublished). We can, therefore, study the kinetics of the reaction between inhibitor and enzyme by plotting the initial activity of the enzyme in the presence of isoascorbate as a function of the time of preincubation with

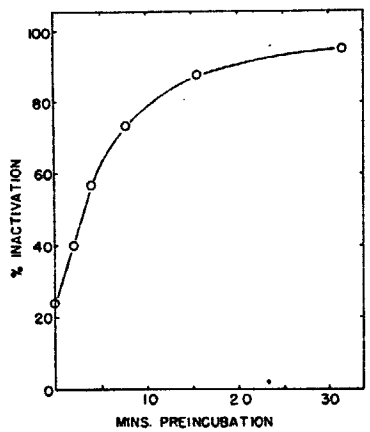


FIG. 2. Course of isoascorbate-induced inactivation of enzyme in spore extract. Twenty micrograms isoascorbate in 0.5 ml. buffer added to 1 ml. spore extract at varying times before adding 6 mg. ascorbate in 0.5 ml. buffer from second side arm.

isoascorbate before addition of substrate. These relations are shown in Fig. 2 in which data from the experiment reported in Fig. 1 are used, the inactivation being calculated from the slopes of the curves during the first 16 min. after addition of ascorbate.

The relationship between isoascorbate concentration and the extent of inhibition was determined both with spore extracts and with toluene-treated spores. Similar results were observed with both types of preparations. Since more extensive data were obtained with treated spores, these alone are reported (Fig. 3). For this experiment aliquots of spores were

incubated with isoascorbate at the indicated concentrations for 1 hr. at 30°C. They were then centrifuged, washed, and resuspended in buffer prior to measurement of activity. (It had been determined previously that the inhibition was not reversed by this treatment, as will be discussed further on.) A linear relation is observed between the degree of inhibition and the isoascorbate concentration. This indicated a stoichiometrical relation between enzyme and inhibitor, and suggested that a

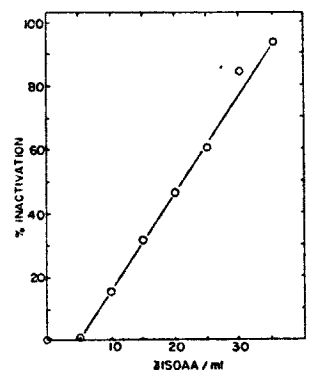


FIG. 3

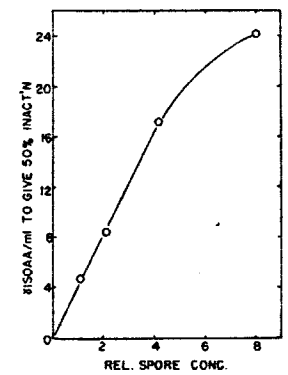


FIG. 4

FIG. 3. Effect of isoascorbate concentration on inactivation of enzymes in toluene-treated spores. Spores incubated ½ hr. with isoAA containing glutathione; centrifuged, washed, and suspended in buffer; activity tested against 3 mg. AA.

FIG. 4. Inactivation by isoascorbate at different spore concentration. Toluene-treated spores incubated 1 hr. with isoAA, centrifuged, washed; activity tested against 3 mg. AA.

comparable relation would obtain between the quantity of enzyme and the concentration of isoascorbate necessary to effect a definite degree of inhibition. Data presented in Fig. 4 show that this is the case. The concentration of isoascorbate required to give 50% inhibition of the enzyme of toluene-treated spores is directly proportional, within limits, to the spore concentration.

To shed further light on the nature of the reaction between enzyme and isoascorbate, the effect of temperature during incubation was determined. The data show the reaction to have a positive temperature coef-

ficient, the rate of inactivation of enzyme by isoascorbate being more rapid as the temperature of preincubation is increased (Fig. 5).

The inactivation is affected very greatly by the pH of the suspensions during incubation with isoascorbate (Table III). At pH 4 only very slight inactivation occurs, while greater inactivation is noted at pH 8 than at pH 6. These data were obtained by treating spores with a low concentration of isoascorbate in buffer at the pH indicated and subse-

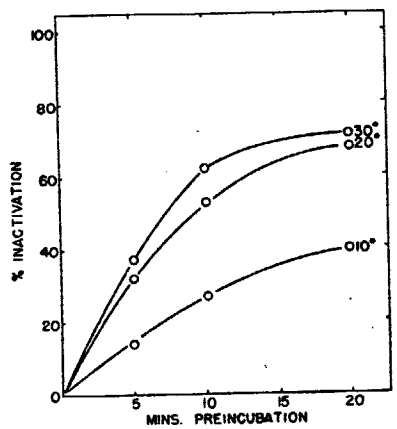


FIG. 5. Effect of temperature and time on isoascorbate-induced inactivation of toluene-treated spores. Spores incubated for varying times at different temperatures with 8 μg. isoAA/ml., centrifuged, washed, and activity tested against 3 mg. AA at 30°C.

quently washing the preparations with buffer at pH 6.3. The spores were finally suspended in this buffer and their activity measured. Failure to get inactivation is not due to reversal during washing as is discussed below. In another experiment a high concentration of isoascorbate (1.0 mg./ml.) was used and added simultaneously with ascorbate at the same concentration. Inactivation does occur under these condition at pH 1. No study of the kinetics of these relations has been made.

It was indicated earlier that the inhibitory action of isoascorbate is irreversible. This was determined for spore extracts by dialysis experi-

ments in which isoascorbate was added to give a concentration of 50 μg./ml. After standing for 1 hr., the preparations were placed in dialysis membranes and dialyzed overnight at about 6°C. against 500 vol. of buffer. No activity was found in these preparations, while controls to which no isoascorbate had been added showed no diminution in activity. Further tests using toluene-treated spores showed that after incubating for 1 hr. with 100 μg. isoascorbate/ml. the inhibition could not be reversed by washing the spores by repeated centrifugation (7×). Additional experiments have shown that even at low concentrations of isoascorbate (5 μg./ml.), where only partial inhibition was effected, the inhibition was not reversed by incubating the treated spores overnight

TABLE III

Effect of pH During Pretreatment with IsoAscorbate on Inactivation of Enzyme in Toluene-Treated Spores

Spores incubated 10 min. with isoascorbate in citrate-phosphate buffer at indicated pH; washed in this buffer and then in phosphate buffer at pH 6.3. Activity measured in this latter buffer.

	Enzyme activity, μl. O_2/10 min.			Inhibition, per cent		
Isoascorbate concn. μg./ml.	pH 4.1	pH 6.0	pH 7.9	pH 4.1	pH 6.0	pH 7.9
0	94	95	94	0	0	0
10	93	58	30	1	39	68
20	89	38	21	5	60	78

on a shaker at 30°C. Treatment of isoascorbate-inactivated spores with ascorbic acid oxidase from squash did not reactivate the enzyme of the spores. While the reaction of isoascorbate with enzyme is markedly affected by pH, the complex formed cannot be reversed by washing the inactivated spores with buffer at various pH levels from 2.7 to 8.0.

The evidence available regarding the nature of the reaction between isoascorbate and enzyme suggested that oxygen might be essential. Preliminary experiments in which spores were incubated anaerobically with isoascorbate in Thunberg tubes indicated a requirement for oxygen. The results were equivocal, however, since oxygen was present while the spores were being washed free from inhibitor. To provide unambiguous data, an apparatus was designed which would enable the entire process to be carried out anaerobically (3). With this apparatus, aliquots of toluene-treated spores were freed of oxygen, and isoascorbate was added anaerobically. After incubation for 1 hr. at 30°C., the suspensions were centrifuged, washed twice with oxygen-free buffer, and then tested

for ascorbic acid oxidase activity. Parallel suspensions were treated under aerobic conditions. The data show (Table IV) that when the enzyme preparation is incubated with isoascorbate in the absence of oxygen, no inactivation occurs.

3. *Further Studies on the Characteristics of the Enzyme*

In the course of the present investigation, in which the reaction was followed at various enzyme and substrate concentrations, it was found that contrary to a previous report (1), the reaction was first order only with certain ratios of concentration of substrate to enzyme. Thus in a plot showing the logarithm of the substrate remaining vs. time, a linear relation can be obtained. If, however, the

TABLE IV

Necessity of Oxygen for Isoascorbate-Induced Inactivation in Treated Spores

Toluene-treated spores incubated 1 hr. with isoascorbate.

Gas	IsoAA μg./ml.	Activity, μl. O_2 @ 10 min.	Inactivation, per cent
H_2	0	80	0
H_2	20	74	7
H_2	200	79	1
Air	0	77	0
Air	20	22	71
Air	200	4	95

ascorbate concentration is decreased, the curve becomes convex; while if it is increased, it bends the other way, becoming concave. Similar relations are noted if the enzyme concentration is changed. Actually the reaction can be interpreted as being bimolecular if the proper ratio of substrate to enzyme is taken.

Obviously two molecular species—oxygen and ascorbate—are involved in the reaction, and while oxygen is present in large excess, the concentration in the liquid phase is much lower than that of ascorbate. Thus in the typical system at 30°C. employing air as the gas phase, and 3 mg. ascorbate per vessel containing a fluid volume of 1.5 ml., the concentrations are 1.14×10^{-2} *M* ascorbate and 2.25×10^{-4} *M* oxygen. Since the reaction rate is not a function of ascorbate concentration under the conditions employed, it was considered possible that oxygen was limiting. Experiments showed this to be the case (Fig. 6). The marked dependence of the rate of oxidation of ascorbate on oxygen tension up to at least 100% O_2 suggested that this might be an additional characteristic differentiating the enzyme from the typical copper-containing as-

corbic acid oxidase. An experiment to check this showed the reaction rates of both systems to be influenced by oxygen in the same manner (Fig. 6).

4. *Ascorbic Acid Oxidase in Mycelium*

A preliminary experiment was carried out to determine whether the mycelium of *M. verrucaria* contained appreciable quantities of an ascor-

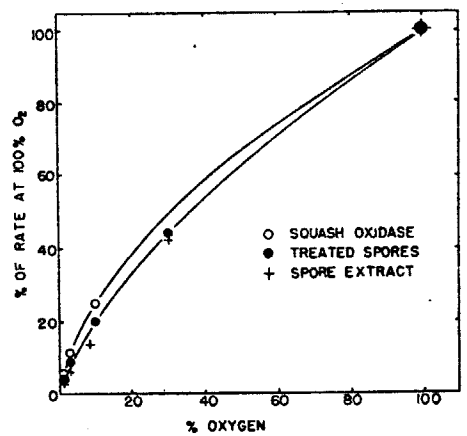
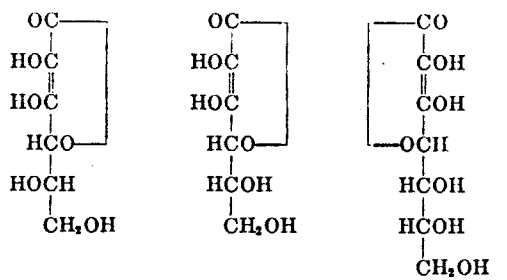


FIG. 6. Effect of oxygen tension on oxidation of ascorbate by toluene-treated spores, spore extract, and ascorbic acid oxidase from squash. Three milligrams ascorbate in 0.5 ml. buffer in side arm, 1 ml. enzyme preparation in vessel; manometers filled with gas by repeated evacuation technique.

bic acid oxidizing enzyme and, if so, whether the enzyme was similar to that present in the spores. Mycelial pellets were grown by inoculating nutrient solution containing mineral salts, sucrose, and yeast extract, and incubating overnight on a shaker. The pellets were washed, suspended in buffer, and their ascorbic acid oxidase activity determined against ascorbic acid and isoascorbic acid. Only the former was oxidized. Furthermore, the isoascorbate inhibited the oxidation of ascorbate. The activity of the mycelium, on a dry weight basis, was less than 10% that of spores of this organism.

Discussion

The specificity of the ascorbic acid oxidizing enzyme of *M. verrucaria* spores and its resistance to poisons of heavy-metal systems clearly distinguishes it from other enzyme systems known to carry out this reaction. The latter enzymes are all copper- or iron-containing proteins and can be grouped into (*a*) those enzymes such as cytochrome oxidase, peroxidase, polyphenol oxidase, and laccase which act indirectly and are consequently not specific toward ascorbic acid; and (*b*) the copper enzyme, ascorbic acid oxidase, which directly oxidizes only ascorbic acid and certain structurally analogous compounds. The ineffectiveness of heavy-metal poisons as inhibitors for the enzyme of *M. verrucaria* spores has been noted previously (1). Data in the present report suggest an absolute specificity of the enzyme for L-ascorbic acid, although the number of analogs tested is too limited to make a positive generalization. The results show that whereas L-ascorbic acid is rapidly oxidized, D-isoascorbate and D-glucoascorbate are not. The structures of these compounds are indicated, as taken from Smith (4):



L-ascorbic acid — D-isoascorbic acid (D-araboascorbic acid) — D-glucoascorbic acid

D-Isoascorbate differs from L-ascorbate only in the configuration about the carbon-5 atom, while D-glucoascorbate has the same configuration as L-ascorbate but has an additional H—C—OH group in the chain. In marked contrast to the specificity of the spore enzyme, investigations by Johnson and Zilva (5) have shown that the unpurified ascorbic acid oxidase of cucumbers oxidizes readily all three of these compounds as well as a number of other analogs. The rates of oxidation were somewhat different, depending upon the configuration and number of carbon atoms.

Further corroborative and extended studies on the specificity of the enzyme of cucumber juice have been made by Snow and Zilva (6) and by Dodds (7).

The oxidation of ascorbate by typical ascorbic acid oxidases and by that in *M. verrucaria* is only slightly affected by substrate concentrations over the range usually employed. Snow and Zilva (6), using partially purified preparations from cucumber, show the rate of oxidation of ascorbate to increase from a rate of about 8 μl. O_2/min. at 0.004 *M* to 10 μl./min. at 0.01 *M* and to about 11 μl./min. at 0.04 *M* ascorbate. Using a highly purified enzyme from squash, Powers *et al.* (2) found only a slight decrease in the rate of oxidation of ascorbate over the range 0.00178–0.0107 *M*. With the *M. verrucaria* enzyme the rate increases only slightly from 0.0038 to 0.06 *M* ascorbate. Apparently the range over which substrate concentration limits the rate of ascorbate oxidation is below 0.002 *M*.

Since the molar concentration of oxygen in the liquid phase is about 1/50 that of ascorbate under the conditions used, it was considered that with the enzyme concentrations employed, the main rate-limiting factor might be oxygen. This proved to be the case, the reaction rate being proportional to the oxygen tension up to 100%. It was thought that this relation might be an additional characteristic differentiating the enzyme from the typical copper-containing ascorbic acid oxidase. Since no pertinent data were encountered in the literature, measurements were made. These showed, however, that both enzymes behaved similarly.

It was reported previously (1) that the course of oxidation of ascorbate by the spore enzyme followed first-order kinetics as had been noted for "typical" ascorbic acid oxidase (8). Data obtained in the present study have shown that first-order kinetics occur only under certain conditions of substrate and enzyme concentration. Under other proportions of substrate to enzyme, the reaction follows a bimolecular course. Thus more involved kinetics must be inferred, as should be anticipated from the phenomenon of reaction inactivation (9) in which the enzyme is inactivated by the substrate.

The behavior of the *M. verrucaria* enzyme toward D-isoascorbate became more interesting when further investigation showed it to be a powerful inhibitor of the enzyme. The close structural relation of this compound to L-ascorbate suggested the inhibition to be competitive. Failure to find the expected relation between degree of inhibition and

relative concentrations of substrate and inhibitor was mitigated partially by data showing that the reaction rate was not greatly influenced by substrate concentration over the range used, and more effectively by demonstration of the irreversible nature of the inhibition.

Further study has shown that isoascorbate reacts chemically with the enzyme. This is indicated by the following facts: (*a*) oxygen is required for the inactivation although the autoxidation products of isoascorbate are not effective; (*b*) there is a stoichiometrical relation between the quantities of enzyme and isoascorbate necessary to effect a given degree of inhibition; (*c*) the isoascorbate-induced inactivation has a positive temperature coefficient; (*d*) the inactivation cannot be reversed by exhaustive washing with buffers at different pH or by dialysis.

The marked dependence of the inactivation upon pH is suggestive of the reaction occurring only with the dissociated acid. At pH 4, ascorbic acid is approximately 50% dissociated (10); presumably isoascorbate has similar characteristics.

It is interesting to compare the inactivation caused by isoascorbate with the reaction inactivation of the enzyme which occurs during the oxidation of ascorbate by purified ascorbic acid oxidase [see Ref. (9) for literature citations] and also by the spore enzyme (1). Ascorbate-induced inactivation proceeds much more slowly, and much higher concentrations are required. Thus with 10 mg. ascorbate/ml. (57×10^{-3} *M*), 50% inactivation is found after about 75 min. at 30°C., whereas with isoascorbate at this concentration, complete inactivation would occur immediately. Even at 20 μg./ml. of isoascorbate (1.2×10^{-4} *M*), 50% inactivation occurs in 30 min. Inactivation by either substance, however, occurs only in the presence of oxygen, has a positive temperature coefficient, and is irreversible.

The action of isoascorbate on the enzyme could be due to combination or to modification by oxidative or other type of reaction. If the latter postulate is correct, it becomes difficult to account for the high specificity of the reaction, since it does not occur with ascorbate or glucoascorbate, both of which have closely similar reactive properties. It is considered more probable that a combination of isoascorbate with the enzyme occurs. It is very tempting to postulate that we have here a stable, ternary complex between enzyme, isoascorbate, and oxygen. We can, perhaps, go one step further and propose that this complex is fundamentally a ternary enzyme–substrate complex. While such com-

plexes have not been demonstrated to occur experimentally, their assumed existence renders certain kinetic data more readily interpretable (11, 12). The stability of the complex with isoascorbate as compared to the fleeting existence of that with ascorbate can be interpreted in accordance with our current understanding of the mechanisms of enzyme specificity. Thus, Pauling (13) postulates the reactive regions of an enzyme to have a structure complementary to that of the substrate in the strained state. Were the structure completely complementary to that of the unstrained substrate, no competition by analogous structures could occur. The stability of the isoascorbate–enzyme complex can then be ascribed to the complementary structure of the active centers to this compound. Possibly the orientation of the hydroxyl on the C-5 atom is the fulcrum which causes the strain in the L-ascorbate molecule when combination with enzyme occurs.

If we assume isoascorbate to react with enzyme only at the reactive sites involved in enzyme–substrate formation, as is indicated by data mentioned previously, we can make an approximate calculation of the number of such centers in the spores. With purified enzyme preparations of known molecular weight such calculation would, of course, have much more significance. Using the data of Fig. 3, 35 μg. isoascorbate/ml. completely inactivated the enzyme of a suspension of spores containing about 4 mg. dry weight of spores/ml. This gives a combining weight with the spores of about 20,000. Since only a small proportion of a spore can be ascorbic acid oxidase, say 1% for purposes of discussion, this would give a combining weight of 200 for the enzyme. This figure, which is probably a maximum value, would force the inference of an inconceivably large number of reactive sites in each molecule of enzyme. We must assume, therefore, that either the reaction does not go to completion, or that isoascorbate reacts with other groups in addition to the reactive sites.

The chemical nature of the enzyme has not been determined. There are no data contradicting the conclusion reached previously that the enzyme is not a heavy-metal protein. No evidence is available with regard to a possible flavoprotein structure. Some experimentation has been carried out to determine whether the enzyme is an aerobic dehydrogenase or an oxidase, using the criteria of Baldwin (14). Since methylene blue cannot act as a hydrogen acceptor in the oxidation of ascorbate, and since the catalase activity of the preparations can be completely

blocked by azide without retarding the oxidation of ascorbate, we must, by definition, consider it as an oxidase in spite of the apparent absence of heavy-metal prosthetic groups.

The physiological role of the ascorbic acid-oxidizing enzyme is still unknown. With the finding of the strong inhibition of the enzyme by isoascorbate it was considered that a useful tool was available for investigating the physiological role of the enzyme. Several types of experiments, however, gave no indication of any effect on metabolic activity by blocking the activity of the enzyme with isoascorbate.

The high specificity of the enzyme toward L-ascorbic acid (vitamin C) indicates its potential value for analytical purposes where a specific method is required. Chemical methods in use cannot discriminate between the isomers or even between other compounds readily oxidized, although separation of L-ascorbate from D-isoascorbate has been shown feasible by paper chromatography (15). Further studies of the substrate specificity of the enzyme using a greater number of compounds is required to permit more precise evaluation of the potentialities of the technique.

Acknowledgments

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Summary

The enzyme of *Myrothecium verrucaria* spores has an indicated absolute specificity for L-ascorbic acid and is irreversibly inactivated by D-isoascorbic acid, oxygen being necessary for this reaction. It is postulated that a stable ternary complex is formed between enzyme, isoascorbate, and oxygen. Studies of the inactivation show it to be markedly affected by pH and to have a positive temperature coefficient. Results of further studies on certain characteristics of the enzyme are reported.

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ADDITIONAL OBSERVATIONS ON THE RATE OF GROWTH OF LACTOBACILLUS CASEI

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In the preceding paper (1) it was shown that the growth rate of *Lactobacillus casei* may be markedly affected by variations in the amount of asparagine present in the medium. This compound may be either stimulatory or inhibitory, depending upon the conditions existing; evidence was presented which indicates that asparagine is closely related metabolically to strepogenin. For example, strepogenin, in the form of trypsinized casein (2), is completely inactivated by mild acid hydrolysis when the test medium does not contain asparagine, whereas its activity is not affected if asparagine is supplied to the organism. Moreover, a culture of *Lactobacillus casei* "trained" by appropriate subculture to grow rapidly without strepogenin was found to require asparagine for rapid development; another "trained" culture grew well in the absence of both, but was markedly inhibited by asparagine. This effect was overcome by trypsinized casein.

As a result of these findings, experiments were undertaken to define more closely the nature of the relationship between asparagine and strepogenin, and to determine whether the rate of growth of *Lactobacillus casei* can be effectively altered by varying the level in the medium of known nutrients other than asparagine.

Experiments along those lines have led to the development of a chemically completely defined medium which is capable of supporting rapid growth of *Lactobacillus casei;* maximum turbidity is obtained in about 24 hours and maximum acid production in about 30 hours. The rate of growth of the organism, under these conditions, is not appreciably affected by trypsinized casein. Upon omission of the appropriate substance, the medium is suitable for the rapid turbidimetric or titrimetric assay of folic acid, biotin, and serine, and should be adaptable for the assay of other compounds required by the organism.

EXPERIMENTAL

In the first experiments conducted, the casein hydrolysate basal medium and inoculum described previously (1) were employed. Since asparagine decreases the rate of growth of *Lactobacillus casei* when added to this medium in the absence of strepogenin, it seemed possible that its inhibitory effect was due to an antagonism between the amide and some constituent

of the basal medium. To test this hypothesis, an excess of each of the amino acids known to exist in casein and each of the vitamins and other supplements employed was added individually to the medium containing asparagine. Serine alone was found to be effective as a growth stimulant under these conditions. Approximately 2.0 mg. of serine, in addition to that present in the casein hydrolysate, are capable of overcoming the inhibition caused by 2.5 mg. of asparagine. However, larger amounts of serine stimulate more rapid growth than is obtained on the original asparagine-free medium. On the other hand, an increase in the serine level in the absence of asparagine is without effect. These results are summarized in Table I.

TABLE I

Effect of Asparagine and Serine* on Rate of Growth of Lactobacillus casei*

Supplement added	Turbidity, 17 hrs.
mg. per tube	*per cent light transmitted*
None†	76
10 mg. serine	76
2.5 mg. asparagine	93, 93
2.5 " " + 2 mg. serine	78, 79
2.5 " " + 10 " "	57, 56

* Throughout the work reported in this paper, L-asparagine, L-glutamic acid, and DL-serine were employed.

† Casein hydrolysate basal medium (1) without asparagine.

In the light of these findings, it appeared advisable to turn our attention to a medium in which the concentration of each constituent could be controlled. An amino acid basal medium patterned after that of Stokes, Gunness, Dwyer, and Caswell (3) was therefore employed in all of the remaining work. As before, the final volume was 10 ml. per tube. Except where otherwise indicated, the tubes containing the basal medium and the various supplements were sterilized by autoclaving at 115° for 12 minutes. After sterilization, the tubes were inoculated and incubated at 37° for the various times specified prior to determining their turbidities or titrating the acid produced. Throughout the work, a light inoculum similar to that described previously (1) was employed, except that 24 hour broth subcultures made daily from agar stabs were used to avoid any possible "training" effect of a week's serial subculture in the broth.

Effect of Amino Acids on Rate of Growth of Lactobacillus casei—The mutually stimulatory effects of serine and asparagine, when added to the basal Medium AA (Table II), were similar to those observed with the casein hydrolysate medium (1). Glutamine was not an effective substitute for asparagine. Moreover, it was found that the rate of growth in the presence

o test this hypothesis, an excess of each of the st in casein and each of the vitamins and other as added individually to the medium containing was found to be effective as a growth stimulant Approximately 2.0 mg. of serine, in addition to hydrolysate, are capable of overcoming the in- g. of asparagine. However, larger amounts of id growth than is obtained on the original as- m the other hand, an increase in the serine level gine is without effect. These results are sum-

TABLE I

nd Serine on Rate of Growth of Lactobacillus casei*

nt added	Turbidity, 17 hrs.
er tube	*per cent light transmitted*
.....	76
.....	76
.....	93, 93
serine.....	78, 79
.....	57, 56

reported in this paper, L-asparagine, L-glutamic acid,

al medium (1) without asparagine.

dings, it appeared advisable to turn our attention e concentration of each constituent could be con- basal medium patterned after that of Stokes, aswell (3) was therefore employed in all of the re- e, the final volume was 10 ml. per tube. Except d, the tubes containing the basal medium and the e sterilized by autoclaving at 115° for 12 minutes. bes were inoculated and incubated at 37° for the rior to determining their turbidities or titrating ughout the work, a light inoculum similar to that was employed, except that 24 hour broth subcul- r stabs were used to avoid any possible "training" nbculture in the broth.

on Rate of Growth of Lactobacillus casei—The mu- of serine and asparagine, when added to the basal were similar to those observed with the casein Glutamine was not an effective substitute for it was found that the rate of growth in the presence

of adequate amounts of serine and asparagine can be further increased by raising the glutamic acid level. Glutamine is capable of replacing glutamic

TABLE II

Composition of Amino Acid Basal Media

Constituent	Concentration	
	Media AA, B	Media B+, BA
	mg. per tube	*mg. per tube*
Amino acid mixture	*	*
L-Asparagine	†	2.5
DL-Serine	†	20.0
L-Glutamic acid	†	20.0
Dextrose (anhydrous)	100.0	100.0
Sodium acetate (anhydrous)	60.0	60.0
Adenine, guanine, uracil, each	0.1	0.1
Salts A		
K_2HPO_4	5.0	10.0
KH_2PO_4	5.0	10.0
Salts B		
$MgSO_4 \cdot 7H_2O$	2.0	4.0
NaCl	0.1	0.2
$FeSO_4 \cdot 7H_2O$	0.1	0.2
$MnSO_4 \cdot 4H_2O$	0.1	0.2
$MnCl_2 \cdot 4H_2O$	0.05	0.25
Ascorbic acid		2.5‡
	γ per tube	*γ per tube*
Riboflavin, thiamine HCl, nicotinic acid, pantothenic acid, each	2.0	4.0
Pteroylglutamic acid	0.02	0.1
Biotin	0.002	0.025
Pyridoxamine	4.0	10.0
Pyridoxine		10.0
Pyridoxal		10.0‡
p-Aminobenzoic acid	0.4	2.0
Inositol		100.0

* Amino acid basal mixture: Per tube 4 mg. of DL-tryptophan, 1 mg. of L-lysine, 2 mg. each of DL-alanine, L-arginine, DL-aspartic acid, L-cystine, glycine, L-histidine, L-hydroxyproline, DL-isoleucine, DL-leucine, DL-methionine, DL-norleucine, DL-phenylalanine, L-proline, DL-threonine, L-tyrosine, and DL-valine.

† The concentrations of L-asparagine, DL-serine, and L-glutamic acid were respectively in Medium AA, 2.5, 2.0, and 2.0 mg., and in Medium B, 2.5, 20.0, and 20.0.

‡ Pyridoxal was omitted from Medium BA and ascorbic acid from Medium B+.

acid when the amide is added at a level of about 0.5 to 1.0 mg. per tube; progressively larger amounts produce decreasing growth responses. These results are illustrated in Table III.

When added to the basal medium supplemented with 20 mg. each of

serine, asparagine, and glutamic acid per tube, none of the other amino acids in amounts up to 20 mg. per tube effected an appreciable increase in growth rate.

Effect of B Vitamins—Although *Lactobacillus casei* grows fairly rapidly on Medium B, which contains 2.5 mg. of asparagine and 20 mg. each of serine and glutamic acid, it was found possible to increase the growth rate

TABLE III

Effect of Serine, Asparagine, and Glutamic Acid on Rate of Growth of Lactobacillus casei

Experiment No.	Amino acids added* per tube	Turbidity, 17 hrs.
		per cent light transmitted
910	20 mg. asparagine, 20 mg. glutamic acid, plus	
	2 mg. serine	94, 95
	10 mg. serine	75, 79
	20 " "	69, 70
	20 mg. serine, 20 mg. glutamic acid, plus	
	0 mg. asparagine	91, 91
	0.5 mg. asparagine	71, 70
	5.0 " "	68, 69
	20.0 mg. asparagine	62, 61
	20 mg. serine, 2.5 mg. asparagine, plus	
	2 mg. glutamic acid	81, 81
	10 mg. glutamic acid	72, 73
	20 " " "	70, 70
916	10 mg. serine, 1 mg. asparagine, 2 mg. glutamic acid, plus	
	No supplement	81, 81
	1 mg. glutamic acid	75, 76
	5 " " "	70, 72
	10 mg. glutamic acid	67, 68
	0.5 mg. glutamine	67, 68
	1 mg. glutamine	71, 71
	5 " "	82, 84
	10 mg. glutamine	88, 89

* The basal medium was Medium AA, minus serine, glutamic acid, and asparagine.

appreciably, but erratically, by the addition of crude materials such as yeast extract, corn steep liquor, and liver. Trypsinized casein was less effective. Since in some experiments the effect of these crude supplements could be partially replaced by increased amounts of biotin plus vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine together, added aseptically), the levels of all of the vitamins and minerals were increased to the values shown under Medium B+ (Table II) to insure that sufficient quantities

amic acid per tube, none of the other amino g. per tube effected an appreciable increase in

hough *Lactobacillus casei* grows fairly rapidly ms 2.5 mg. of asparagine and 20 mg. each of was found possible to increase the growth rate

TABLE III

d Glutamic Acid on Rate of Growth of Lactobacillus casei

mino acids added* per tube	Turbidity, 17 hrs.
	per cent light transmitted
ne, 20 mg. glutamic acid, plus	
	94, 95
	75, 79
	69, 70
mg. glutamic acid, plus	
ne	91, 91
agine	71, 70
	68, 69
agine	62, 61
5 mg. asparagine, plus	
c acid	81, 81
nic acid	72, 73
"	70, 70
mg. asparagine, 2 mg. glutamic acid,	
nt	81, 81
nc acid	75, 76
"	70, 72
nic acid	67, 68
nine	67, 68
ine	71, 71
	82, 84
nine	88, 89

edium AA, minus serine, glutamic acid, and asparagine.

lly, by the addition of crude materials such as liquor, and liver. Trypsinized casein was less experiments the effect of these crude supplements ed by increased amounts of biotin plus vitamin , and pyridoxamine together, added aseptically), amins and minerals were increased to the values + (Table II) to insure that sufficient quantities

for optimum growth were present. Inositol was also included in the modified medium.

The relative growth-stimulating effects of corn steep liquor solids, yeast extract (Difco), and trypsinized casein, when added to the basal medium containing all of these supplements (Medium B+), are shown in Fig. 1.

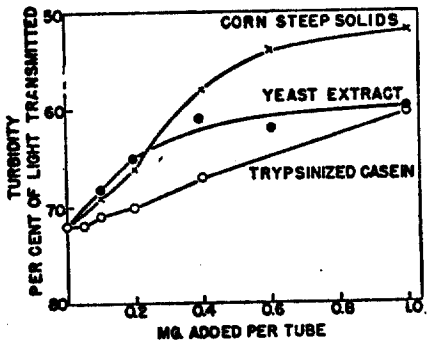

FIG. 1. Effect of crude supplements on the growth of *Lactobacillus casei;* basal Medium B+, 17 hours incubation.

TABLE IV

Effect of Added Reducing Agents on Growth Rate of Lactobacillus casei (Basal Medium B+)

Experiment No.	Supplement added per tube	Supplement sterilized by filtration and added aseptically	Supplement sterilized by autoclaving with medium
		per cent light transmitted	*per cent light transmitted*
1112	None	79	79
	0.5 mg. ascorbic acid	65	65
	1.0 " " "	56	56
	2.5 " " "	45	45
	1.0 " thioglycolic acid	66	60
	2.5 " " "	54	56
	2.5 " cysteine	70	70
23	None		59, 58
	2.5 mg. ascorbic acid		39, 40
	2.5 " *d*-isoascorbic acid		36, 36
	3.0 " corn steep solids		34
	3.0 " trypsinized casein		41

Turbidities were measured after 17 hours incubation. The relatively slight activity of the trypsinized casein suggests that the stimulation produced by corn steep solids and yeast extract is due largely to some factor other than strepogenin. Essentially the same results were obtained when pyridoxal was omitted, and the tubes containing basal medium and all of the

supplements sterilized by autoclaving. This procedure was therefore followed in the remaining experiments, except those in Table IV.

Effect of Other Known Compounds—Since the growth rate of *Lactobacillus casei* on basal Medium B+ can be increased by the above-mentioned crude materials, it appeared advisable to test the possible effect of an additional series of known compounds. It was found that increasing by 5-fold the mineral level of the medium had no effect; xanthine was inhibitory at levels above 10 γ; hypoxanthine, guanosine, cytidylic acid, and yeast nucleic acid at levels up to 50 γ per tube, nicotinamide (0.5 to 3.0 γ per tube), and sulfuric acid-hydrolyzed casein (12.5 to 125 mg. per tube) were without effect. However, a mixture of 5 mg. each of fumaric, succinic, and ascorbic acids and sodium ethyl oxalacetate produced a definite response in growth rate. This was found to be due to the ascorbic acid content of the mix-

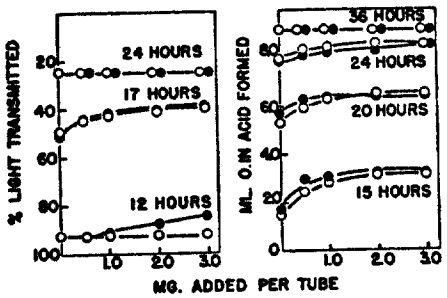

FIG. 2. Effect of trypsinized casein and corn steep liquor upon the rate of growth of *Lactobacillus casei*. Basal Medium BA; ○, trypsinized casein; ●, corn steep liquor solids.

ture. As might be anticipated, the effect of this compound appears to be due to its reducing properties rather than to a possible specific vitamin effect. The growth-accelerating effect of ascorbic acid is compared with that of other reducing agents in Table IV. It is apparent that cysteine is not effective at the levels used, while thioglycolic acid is less effective than ascorbic acid, and *d*-isoascorbic acid is equally potent.

Effect of Crude Materials on Rate of Growth of Lactobacillus casei on Improved Synthetic Medium BA—Since relatively rapid growth of *Lactobacillus casei* is attained on basal Medium BA (containing the ascorbic acid supplement), a careful check was made on the possible effect on the growth rate of the organism when trypsinized casein, a known source of strepogenin, is added. Growth response curves, both turbidimetric and titrimetric, for *Lactobacillus casei* with graded amounts of trypsinized casein and corn steep liquor were determined at varying incubation times. The tubes containing basal medium, ascorbic acid, and supplements were sterilized by autoclav-

utoclaving. This procedure was therefore fol-
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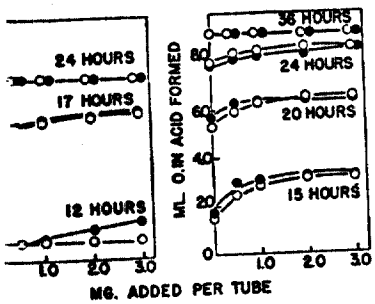

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ing. Turbidities were determined after 8, 12, 14, 16, 17, 20, and 24 hours incubation and acid production in replicate sets of tubes after 15, 20, 24, 36, and 45 hours. The results of these experiments are summarized in Fig. 2. The curves for some of the time intervals are omitted for the sake of clarity. In addition, the rates of growth (turbidity) and of acid production by the organism in the basal Medium BA without crude supplements

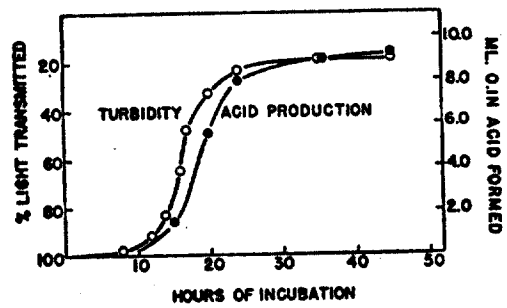

FIG. 3. Rate of growth of *Lactobacillus casei* on basal Medium BA; no supplements added.

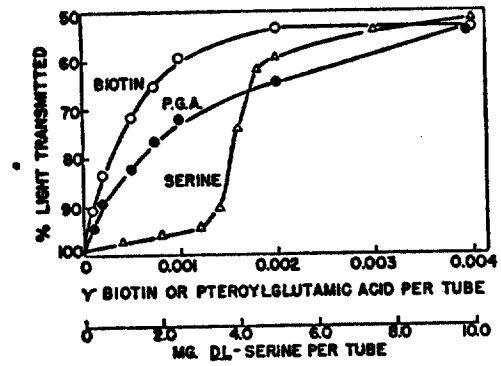

FIG. 4. Growth response of *Lactobacillus casei* to biotin, pteroylglutamic acid, and DL-serine. Basal Medium BA minus the compound tested; 17 hour incubation period.

are plotted in Fig. 3. It is of interest that there is only about a 3 hour lag between turbidity and acid production.

Use of Improved Lactobacillus casei Medium (BA) As Basal Medium for Amino Acid and Vitamin Assays—Medium BA gives constant and reproducible rapid growth which is not appreciably affected by the addition of crude materials likely to contain "non-specific" stimulatory substances. It should, therefore, be well suited, upon the omission of the appropriate nutrient, for the rapid and accurate assay of those vitamins and amino

acids required by *Lactobacillus casei*. A few tests along these lines were carried out. Growth response curves (17 hour turbidity) for DL-serine, pteroylglutamic acid, and biotin are illustrated in Fig. 4. Similar curves were obtained by titrating the acid formed after 22 hours. While we have not had the opportunity to carry out assays of various source materials for comparison with literature values, it is felt that the present method should prove satisfactory, since the standard curves shown in Fig. 4 are highly reproducible. It appears probable that a more practical basal medium for assays of some of the vitamins could be developed with hydrolyzed casein instead of purified amino acids as the nitrogen source.

DISCUSSION

The chief factor limiting the *rate* of growth of *Lactobacillus casei* in a casein hydrolysate or amino acid medium of the usual type is presumably the rate of synthesis of strepogenin. It follows, therefore, that the increase in growth rate which is observed when additional quantities of serine, asparagine, and glutamic acid are supplied to the organism is due to an increase in the rate of strepogenin synthesis. These amino acids may function directly as precursors of strepogenin; glutamic acid and possibly serine have been postulated as components of the strepogenin molecule (4). Since DL-serine was employed in the present work, the possible rôle of D-serine remains to be investigated. Evidence presented previously (1) indicates that there is a close metabolic relationship between asparagine and strepogenin; asparagine may represent a component of the strepogenin molecule or alternatively may serve as an amide donor. The latter interpretation is consistent with the observation that mild acid hydrolysis destroys strepogenin activity in the absence of added asparagine, but is without effect when the amide is supplied to the organism (1).

The effectiveness of *l*-ascorbic acid and *d*-isoascorbic acid in further increasing the rate of growth of *Lactobacillus casei* is presumably due to their reducing effect. It appears unlikely that an appreciable fraction of the growth stimulation produced by the addition of trypsinized casein to the original medium can be attributed to a similar effect, since the amounts added are small.

The 17 hour growth response curve for DL-serine (Fig. 4) is of interest in that the stimulatory effect of this amino acid under these conditions is not observed until amounts substantially larger than the requirement for full growth upon prolonged incubation are added. Thus, 2 mg. per tube are adequate if growth is allowed to proceed for 72 hours, whereas about 7 mg. per tube are required for an optimum *rate* of growth. It is possible that serine plays two distinct rôles in the nutrition of *Lactobacillus casei*: its direct utilization as a cell protein component and also as a possible precursor of strepogenin.

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Some difficulty has been reported in the use of *Lactobacillus casei* for microbiological assays because of the effect of non-specific stimulatory substances on the growth of the organism (5). It is believed that improvement of basal assay media along the lines indicated in the present paper would resolve these difficulties to a considerable extent.

SUMMARY

A chemically defined complete medium has been developed which permits the rapid growth of *Lactobacillus casei*. Essentially maximum turbidity is obtained at about 24 hours and maximum acid production at about 30 hours.

The organism does not grow at an appreciably more rapid rate when trypsinized casein, a known source of strepogenin, is added to the improved medium.

The pronounced increase observed in the growth rate of the organism when the levels of asparagine, serine, and glutamic acid are increased is consistent with the view that these amino acids may serve directly or indirectly as precursors of strepogenin. Serine is stimulatory in an amount substantially greater than the requirement for growth upon prolonged incubation.

The improved medium, upon omission of the appropriate nutrient, is capable of serving as a basal medium for the rapid turbidimetric or titrimetric assay of substances required for the growth of *Lactobacillus casei*.

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constant was higher than could be accounted for on the basis of the incidence of spontaneous tumors in this strain. The hypothesis implies an average of more than 3 tumors at the zero dose, whereas the observed average was .29. This increased response is even more difficult to explain than the decreased response shown for the single-action hypothesis. The two-action hypothesis implies that the curve is concave upward, which would give a rapidly increasing number of tumors at the larger doses. Instead, at the higher doses the curve probably would be found to flatten out. Since there is also a time element involved if the actual response curve is sigmoid, observing the tumors earlier might give an upward curve, whereas later observations might give a downward curve. This requires further investigation.

Data on pulmonary tumors induced in mice with methylcholanthrene by Shimkin and McClelland (*8*) were examined to see if the linear relationship between the number of nodules and the dose was also present in their experiment. Such a linear relationship could not be established, there being significant departure from linearity when the χ^2 test was used. However, there were not as many animals per group in their experiment as in the groups reported herein, and the technique used in counting was not the same for the two experiments. In their experiment the nodules were counted in lungs that previously had been fixed in Tellyesniczky's fluid, and apparently the counting was done without the aid of the dissecting microscope. Their data did not fit a parabolic curve such as reported by Charles and Luce-Clausen either.

It was possible, however, to estimate the "net disposal" constant (k) for Shimkin and McClelland's data such as was estimated from our own experiment. Their data were in three series, with observations made after 8, 13, and 18 weeks. For these series, k was .047 mg, .040 mg, and .047 mg methylcholanthrene, respectively. These constants are more nearly alike than would have been expected from the variance of k which is implied by the mathematics.

Although the data of Shimkin and McClelland do not support, they do not oppose our own data, which with the analysis thereof would fit the postulate that the induction of pulmonary tumors in the mouse is the result of a single change in the cell. If this were a genic change it would be assumed to be a dominant mutation.

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Ascorbic Acid and the Oxidation of Tyrosine

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Research Service, Third (New York University) Medical Division, Goldwater Memorial Hospital, New York, Section on Chemical Pharmacology, National Heart Institute, National Institutes of Health, USPHS, Bethesda, Maryland, and Division of Biochemistry, University of California School of Medicine, Berkeley

Many lines of evidence have suggested that ascorbic acid is involved in the biological oxidation of tyrosine. Studies with rat liver homogenate preparations (*1*) and acetone powder extracts (*2*) have indicated that the effect of ascorbic acid in the conversion of tyrosine to acetoacetic acid is primarily upon the first oxidative step of this metabolic pathway, the oxidation of *p*-hydroxyphenylpyruvic acid. Recently it has been proposed that ascorbic acid is a cofactor for the enzyme catalyzing this oxidative step (*3*). In the present communication, evidence will be presented that ascorbic acid may have a less specific role and can be replaced by a number of structurally unrelated compounds that are susceptible to oxido-reduction.

In experiments with extracts of liver acetone powder of rat, rabbit, and dog, it has been reported that tyrosine, *p*-hydroxyphenylpyruvic acid, 2,5-dihydroxyphenylpyruvic acid, and homogentisic acid oxidized enzymatically to acetoacetic acid (*2, 4*). The addition of ascorbic acid to this preparation increases the oxidation of tyrosine or *p*-hydroxyphenylpyruvic acid but has no effect upon the oxidation of 2,5-dihydroxyphenylpyruvic acid and homogentisic acid (*2*). The effect of ascorbic acid on the oxidation of *p*-hydroxyphenylpyruvic acid can be studied by using either *p*-hydroxyphenylpyruvic acid as the substrate or by using tyrosine with α-ketoglutarate to generate *p*-hydroxyphenylpyruvic acid via the tyrosine transaminase system present in the extract. The effect of ascorbic acid was established by measuring the rate of disappearance of substrate and by following the reaction manometrically in the Warburg apparatus. When 10 μM of L-tyrosine was used as the substrate with the liver extract preparation, the addition of ascorbic acid increased the oxidation of tyrosine only when α-ketoglutarate was also present, as shown in Fig. 1. A concentration of 0.001 *M* (4 μM) ascorbic acid was found to be sufficient to produce a maximal stimulatory effect upon the oxidation. Further increase in the concentration of ascorbic acid did not increase the initial rate of oxygen uptake, nor did it alter the products of the reaction. Suboptimal concentrations of ascorbic acid resulted in less tyrosine being converted to acetoacetic acid.

The preparation of the acetone powder removes part of the ascorbic acid orginally present in the tissue. The residual amount remaining in the crude powder extract is sufficient, however, to maintain appreciable oxidative activity. Dialysis of the crude

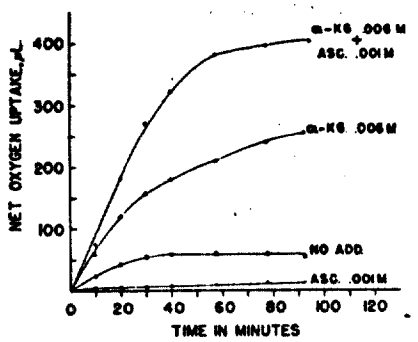

FIG. 1. The stimulation of L-tyrosine oxidation by ascorbic acid (ASC) in the presence of α-ketoglutarate (α-*KG*). Flask contents: 2.0 ml rabbit liver powder extract; 1.0 ml pyrophosphate buffer; 0.3 ml α-ketoglutarate (20 µM) or 0.3 ml of H_2O. The side arms contained 10 µM L-tyrosine in 0.5 ml phosphate buffer (buffer alone was used in the control flasks), and 0.2 ml ascorbic acid (4.0 µM). Total volume, 4.0 ml. Both α-ketoglutarate and ascorbic acid were omitted in the no-addition flasks (*No ADD.*).

powder extract greatly reduces the ability to oxidize *p*-hydroxyphenylpyruvic acid, but this activity is nearly completely restored by the addition of ascorbic acid to the dialyzed extract system.

Considerably less than stoichiometric amounts of ascorbic acid are needed for the oxidation of tyrosine. Titration with 2,6-dichlorophenolindophenol at the end of the incubation period has shown that nearly half the added ascorbic acid remains in the reduced form. Since the titration values are the same for the control flasks and the ones in which tyrosine was oxidized, it appears that no net consumption of ascorbic acid is required for the oxidation of tyrosine.

Although these results are in agreement with the theory that ascorbic acid acts as a cofactor for the enzyme system catalyzing the oxidation of *p*-hydroxyphenylpyruvic acid, the data presented below suggest that ascorbic acid may act in a less specific manner.

Knox (*1*) observed in experiments with the rat liver homogenate preparation, that D-isoascorbic acid also increases the oxidation of tyrosine. We have tested several compounds using the acetone powder extract preparation and have found that D-isoascorbic acid, D-ascorbic acid, and hydroquinone are just as effective as L-ascorbic acid on a molar basis. Homogentisic acid, *p*-aminophenol, *p*-phenylenediamine, and 2,6-dichlorophenolindophenol also increase tyrosine oxidation but are less effective than ascorbic acid. On the other hand, catechol, resorcinol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylalanine, dihydroxymaleic acid, cysteine, and glutathione are unable to replace ascorbic acid or to supplement a suboptimal concentration of ascorbic acid. The oxidized forms of ascorbic acid or hydroquinone (dehydroascorbic acid or quinone) are nearly as effective as the reduced forms. This would be expected if these compounds undergo cyclic oxidation and reduction during the oxidation of tyrosine.

The observation that several compounds are able to stimulate the oxidation of *p*-hydroxyphenylpyruvic acid suggests that the requirement is one for a compound having the proper oxidation-reduction potential. Whether these compounds found to be active in place of ascorbic acid function by protecting the small amount of ascorbic acid present in the powder extract or completely replace ascorbic acid in this system cannot be determined without further purification of the enzyme system involved.

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Manuscript received July 8, 1952.

Comments and Communications

Zoological Nomenclature

NOTICE is hereby given that, as from June 29, 1953, the International Commission on Zoological Nomenclature will start to vote on the following cases involving the possible use of its plenary powers for the purposes specified against each entry. Full particulars of these cases were published on Dec. 29, 1952, in the *Bulletin of Zoological Nomenclature* in Double-Part 4/5 of Vol. 9. (1) *Astacus Fabricius*, 1775 (Class Crustacea, Order Decapoda), validation of (correction of an error in Opinion 104); (2) *Favus* Lanchester, 1900 (Cl. Crustacea, Ord. Decapoda), validation of (correction of an error in Opinion 73); (3) *flavipes* Olivier, 1795, *Dytiscus* (Cl. Insecta, Ord. Coleoptera), validation of, by the suppression of *flavipes* Fabricius, 1792, *Dytiscus*.

Comments on the above cases should be sent as soon as possible to the undersigned.

FRANCIS HEMMING
International Commission on Zoological Nomenclature
28 Park Village East, Regent's Park
London, N.W. 1, England

Scientific Conferences and Papers

IT SEEMS worth while at this time, when so many conferences on such a variety of subjects are scheduled, to review the fundamental purposes of a scientific conference and the methods of best achieving these ends.

The principal objective of a scientific conference

Arginine hydrochloride (L)/Daunomycin

BRICHO	ORGANISM	ASSAY SYSTEM	TREATMENT CONDITIONS	CONCENTRATION	TIME	TEMP	BIOLOGICAL EFFECT	AUTHOR'S REMARKS
007041	Human leukocytes (IN VITRO)	Mitotic index; chromosome aberrations	Agent added to 60h-old leukocyte cultures obtained from females; vinblastine added during last 3 h of culture	0.16% (arginine) + .01 or .02 microg /ml (daunomycin)	12h		Increase (synergism) in both mitotic index and frequency of chromosome aberrations (26 to 71% aberrant cells, 0.5 to 3.4 aberrations/cell); dicentrics; double fragments; gaps; chromatid and isochromatid exchanges; intra- and inter-chromosomal rearrangements	Combination of daunomycin with arginine produces chromosome gaps by affecting thymidylate activity

Ascorbic acid

BRICHO	ORGANISM	ASSAY SYSTEM	TREATMENT CONDITIONS	CONCENTRATION	TIME	TEMP	BIOLOGICAL EFFECT	AUTHOR'S REMARKS
010902	Plant (ALLIUM CEPA)	Abnormal anaphase and telophase (chromosome breaks)	Roots soaked in aqueous solution of agent; 5 h post-treatment in tap water and fixed	10(-2) M	2 h		No significant increase in chromosome damage or mitotic disturbances	Protects against radiation damage (100r)
11059	Cow spleen DNA	Decrease in DNA viscosity	Agent added to 0.15% DNA solution	6.76 x 10(-2) or 1.35 x 10(-1) M	0 or 24 h		Moderate reduction in DNA viscosity	

Asparagine

BRICHO	ORGANISM	ASSAY SYSTEM	TREATMENT CONDITIONS	CONCENTRATION	TIME	TEMP	BIOLOGICAL EFFECT	AUTHOR'S REMARKS
010902	Plant (ALLIUM CEPA)	Abnormal anaphase and telophase (chromosome breaks)	Roots soaked in aqueous solution of agent; 5 h post-treatment in tap water and fixed	10(-2) M	2 h		No significant increase in chromosome damage or mitotic disturbances	Protects against radiation damage (100r)
009270	Plant (ALLIUM CEPA)	Abnormal metaphase (root tips)	3 to 5 cm roots immersed in agent solution pH 6; bulbs and roots irradiated 100 R; immersed in tap water 5 h, root tips fixed and stained	10(-2) M	2 h		No significant change in aberration frequency	Slight radioprotection

Benzaldehyde

BRICHO	ORGANISM	ASSAY SYSTEM	TREATMENT CONDITIONS	CONCENTRATION	TIME	TEMP	BIOLOGICAL EFFECT	AUTHOR'S REMARKS
010843	Plant (ALLIUM CEPA)	Cytological abnormalities (mitosis)	Roots immersed in agent solution; root tips fixed and stained	5 x 10(-6) to 1.8 x 10(-5) M	4 h		Significant increase in multipolar mitosis; low threshold value	Effect may result from intracellular conversion of aldehyde to alcohol

CLXX. THE RELATION BETWEEN THE RATE OF ENZYMIC OXIDATION AND THE STEREO-CHEMICAL STRUCTURE OF ASCORBIC ACID AND ITS ANALOGUES

By SYDNEY WALGATE JOHNSON
and SYLVESTER SOLOMON ZILVA[1]

From the Division of Nutrition, Lister Institute, London

(*Received 29 June 1937*)

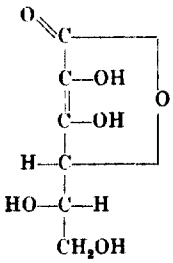
The experiments to be described in this communication are the outcome of a chance observation made in connexion with a previous inquiry [Johnson & Zilva, 1937]. In that investigation on the reversible oxidation of *l*-ascorbic acid by vegetable enzymes, a differentiation was made between oxidation by enzymes of the type described by Szent-Györgyi [1930; 1931], present in cabbage, cauliflower, cucumber etc., which oxidize the acid directly and by the phenolases, such as exist in the potato and apple, which cause the oxidation of the acid indirectly. The latter was shown in the first place [Zilva, 1936] to be able to oxidize *d*-gluco-ascorbic acid (3-keto-*d*-glucoheptonofuranolactone) which is not antiscorbutically active. Johnson & Zilva [1937] have since demonstrated that the enzyme which oxidizes *l*-ascorbic acid directly also oxidizes this homologue. In the experiments designed to prove this fact, *l*-ascorbic acid was used as a substrate in a control series of determinations in order to ensure that the enzyme was fully active. It was then observed that the oxidation of the latter proceeded at a much higher rate than that of *d*-gluco-ascorbic acid. On repeating the experiment it was found that the observation was reproducible. As a consequence of this and of the fact that a relationship has already been established between stereochemical structure and retention in the animal body on the one hand, and the antiscorbutic activity of the analogues of ascorbic acid on the other [Zilva, 1935, 1, 2], it was decided to study the kinetics of the direct enzymic oxidation of these compounds in detail.

Technique

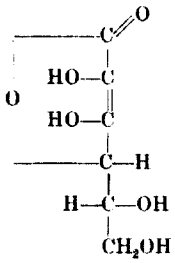
On account of the great accuracy of the method and of the shortage of experimental material recourse was made to the determination of the rates of oxidation of ascorbic acid and its various analogues by means of a Warburg respirometer. According to the purity of these compounds, 10–12 mg. of the substance were dissolved in 4·0 ml. of *M*/15 KH_2PO_4 (made up in quartz-distilled water) *p*H 6·0, the optimum for the activity of the enzyme. Immediately before beginning the experiment 0·5 ml. of each solution was titrated with 1·14*N*/200 2:6-dichlorophenolindophenol in order to ascertain the actual concentration of the ascorbic acid compound. 1 ml. of these solutions, equivalent to about 2·5 mg. of ascorbic acid was pipetted into a Warburg respirometer flask of about 16 ml. capacity. The enzyme was introduced into the side limb of the flask as 1·0 ml. of cucumber juice prepared by the procedure already described [Johnson & Zilva, 1937]. In

[1] Member of the Scientific Staff, Medical Research Council.

the corresponding controls the enzyme solution was replaced by 1·0 ml. of $M/15$ KH_2PO_4. In order to ensure that none of the absorbed oxygen was due to the enzyme solution itself, 1·0 ml. of buffer and 1·0 ml. of cucumber juice were employed as an additional control. It was in fact invariably found that the flask containing this mixture, as well as the flasks containing the control ascorbic acid solutions or solutions of its analogues, actually behaved in the same manner as the thermobarometer which contained 2·0 ml. of the buffer solution. The determinations were made at 26°. At the end of the experimental period the contents of the flasks were acidified with 0·5 ml. of glacial acetic acid in order to arrest the action of the enzyme and titrated with indophenol. Only in the case of the analogues where the enzymic reaction proceeded slowly was any residue of the compound found. The controls seldom showed any significant fall from the original titre.



l-Ascorbic acid



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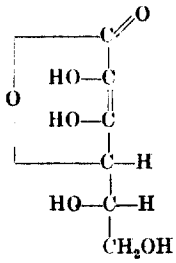
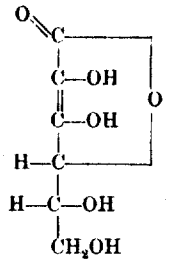
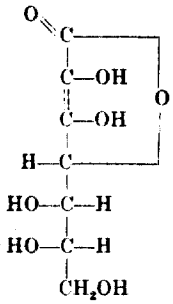


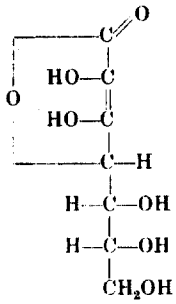
l-Arabo-ascorbic acid



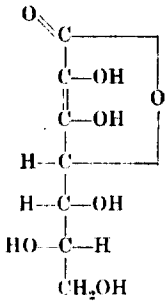
d-Arabo-ascorbic acid



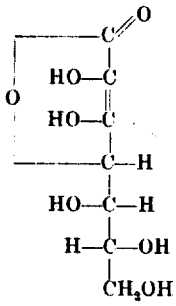
l-Gluco-ascorbic acid



d-Gluco-ascorbic acid



l-Galacto-ascorbic acid



d-Galacto-ascorbic acid

Exp. 1. In this original experiment the precipitated enzyme of the marrow was used and the oxidation of *l*-ascorbic acid and of *d*-gluco-ascorbic acid was followed up by titration with indophenol. Into each of a series of tubes were introduced a quantity of enzyme equivalent in activity to 1·0 ml. of the original marrow juice and 0·7 mg. of *l*-ascorbic acid or the equivalent quantity of its homologue dissolved in 2·0 ml. of $M/15$ KH_2PO_4, *p*H 6·0. At specified times 0·5 ml. of glacial acetic acid was added to the tubes to stop the action of the enzyme and the resulting solution was titrated with 1·14 $N/1000$ indophenol. It will be seen from Table I that the oxidation of *d*-gluco-ascorbic acid proceeded much more slowly than that of *l*-ascorbic acid. The latter compound was fully dehydrogenated by the enzyme in 2–3 hr. whilst only about two-thirds of the former was oxidized in 4 hr. under the same conditions. As already mentioned this result was found to be reproducible.

Table I. *A comparison of the rates of oxidation of* l-*ascorbic and* d-*gluco-ascorbic acids by marrow juice ascorbic acid oxidase*

Time in hr.	mg. *l* ascorbic acid in 2·0 ml. of test solution		mg. *d*-gluco-ascorbic acid in 2·0 ml. of test solution	
	l-Ascorbic acid in *M*/15 KH_2PO_4 *p*H 6·0	*l*-Ascorbic acid and precipitated marrow juice enzyme in *M*/15 KH_2PO_4 *p*H 6·0	*d*-Gluco-ascorbic acid in *M*/15 KH_2PO_4 *p*H 6·0	*d*-Gluco-ascorbic acid and precipitated marrow juice enzyme in *M*/15 KH_2PO_4 *p*H 6·0
0	0·70	0·70	0·82	0·82
0·25	0·70	0·50	0·82	0·74
0·50	0·70	0·43	0·82	0·67
1·0	0·70	0·36	0·82	0·56
1·5	0·70	0·32	0·82	0·49
2·0	0·70	0·15	0·82	0·42
3·0	0·70	0·00	—	—
4·0	—	—	0·82	0·24

Note. 0·70 mg. *l*-ascorbic acid and 0·82 mg. *d*-gluco-ascorbic acid possess equal indophenol-reducing capacities, viz. 7·0 ml. 1·14 *N*/1000 2:6-dichlorophenolindophenol.

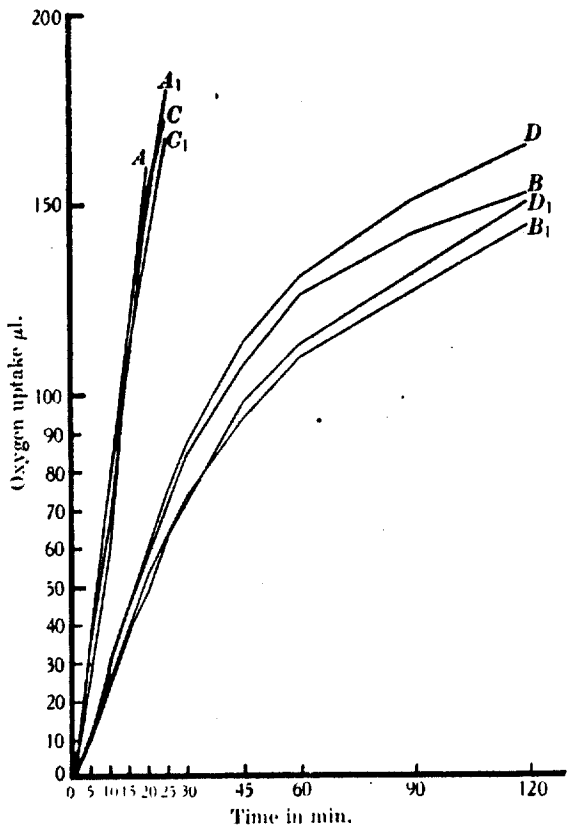


Fig. 1.

A, *l*-ascorbic acid; *B*, *d*-ascorbic acid; *C*, *d*-arabo-ascorbic acid; *D*, *l*-arabo-ascorbic acid — 1st determination.

A_1, *l*-ascorbic acid; B_1, *d*-ascorbic acid; C_1, *d*-arabo-ascorbic acid; D_1, *l*-arabo-ascorbic acid — 2nd determination.

Exp. 2. Since determinations could only be carried out on four compounds at a time in the Warburg respirometer, the rates of oxidation of the following compounds were studied in this experiment. *l*-Ascorbic acid (3-keto-*l*-gulonofuranolactone), *d*-arabo-ascorbic acid (3-keto-*d*-gluconofuranolactone), *d*-ascorbic acid (3-keto-*d*-gulonofuranolactone) and *l*-arabo-ascorbic acid (3-keto-*l*-gluconofuranolactone). All these compounds contain six carbon atoms: whilst in the first two the ring engages the hydroxyl group attached to the fourth carbon atom on the right of the carbon chain, in the last two this takes place to the left. The experiment was carried out twice on consecutive days, the same preparation of the enzyme being used. The results of both series of determinations are represented graphically in Fig. 1. They show that in spite of the slight deterioration in the activity of the enzyme, as found on the following day, the compounds fall into two distinct groups as regards their rates of oxidation. These groups are characterized by the position of the oxygen bridge in the respective analogues. Thus *l*-ascorbic acid and *d*-arabo-ascorbic acid, with the oxygen bridge to the right of the carbon chain, were oxidized much more rapidly than their respective enantiomorphs.

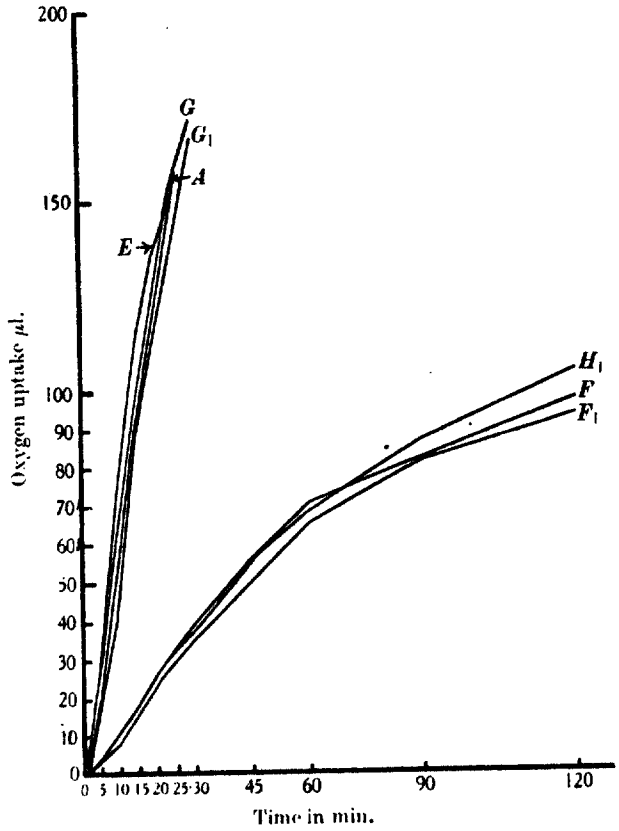


Fig. 2.

E, *l*-gluco-ascorbic acid, *F*, *d*-gluco-ascorbic acid, *G*, *l*-galacto-ascorbic acid } 1st determination.

A, *l*-ascorbic acid, F_1, *d*-gluco-ascorbic acid, G_1, *l*-galacto-ascorbic acid, H_1, *d*-galacto-ascorbic acid } 2nd determination.

Exp. 3. The rates of oxidation of seven-carbon analogues were compared with that of *l*-ascorbic acid in this experiment. The following were investigated: *d*-gluco-ascorbic acid (3-keto-*d*-glucoheptonofuranolactone), *d*-galacto-ascorbic acid (3-keto-*d*-galactoheptonofuranolactone), *l*-gluco-ascorbic acid (3-keto-*l*-glucoheptonofuranolactone) and *l*-galacto-ascorbic acid (3-keto-*l*-galactoheptonofuranolactone). In a first determination these homologues were compared amongst themselves. In a second determination *l*-galacto-ascorbic acid, *d*-galacto-ascorbic acid and *d*-gluco-ascorbic acid were compared with *l*-ascorbic acid. In both determinations the same preparation of the enzyme was employed. All the results are given graphically in Fig. 2. Here the relationship between the position of the oxygen ring with respect to the carbon chain and the rate of oxidation is similar to that observed in the previous experiment. Thus the oxidation of the *l*-forms, which have the oxygen ring to the right of the carbon chain, proceeds much more rapidly than that of the corresponding *d*-forms.

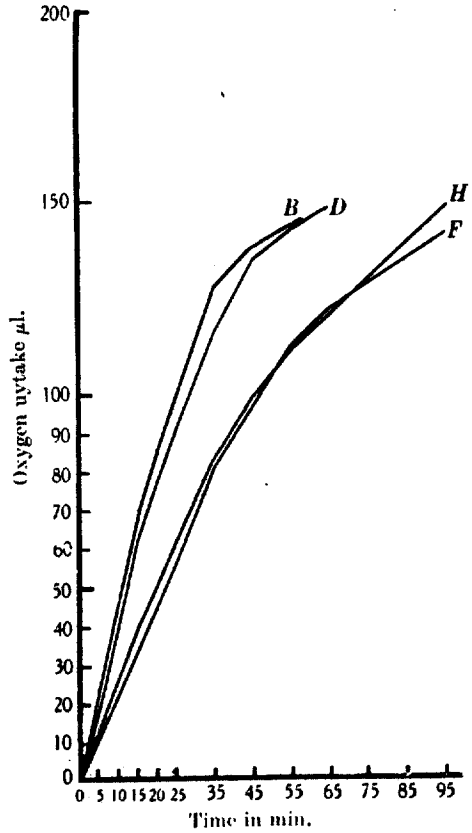


Fig. 3.

B, *d*-ascorbic acid. *F*, *d*-gluco-ascorbic acid.
D, *l*-arabo-ascorbic acid. *H*, *d*-galacto-ascorbic acid.

Exp. 4. By comparing Figs. 1 and 2 it will be seen that besides the fact that the rates of oxidation of *l*-ascorbic acid and its analogues were influenced by the position of the oxygen ring with respect to the carbon chain, the number of atoms in the molecule also seems to have had an influence on the kinetics of the

reaction. For instance in the group of the more slowly oxidizable compounds, in which the hydroxyl group is engaged by carbon atom four to the left of the carbon chain, the seven-carbon compounds appeared to be dehyrogenated less rapidly than the corresponding six-carbon analogues. The comparison of the two experiments becomes more significant when the fact is taken into consideration that the experiments were carried out on consecutive days using the same enzyme preparation. It was, however, desirable to perform a separate experiment in order to obtain confirmation of this phenomenon. The rate of oxidation of the six-carbon compounds, *d*-ascorbic acid and *l*-arabo-ascorbic acid was therefore compared with that of the seven-carbon analogues *d*-gluco-ascorbic acid and *d*-galacto-ascorbic acid. It will be seen from Fig. 3 that the difference in the rates of oxidation previously observed is confirmed by this experiment.

Discussion

The results of the investigation are summarized in Fig. 4. The curves for *l*-ascorbic acid, *d*-arabo-ascorbic acid, *d*-ascorbic acid and *l*-arabo-ascorbic acid are constructed from the averages of the duplicates shown in Fig. 1 (Exp. 2). The curve for *l*-gluco-ascorbic acid is the same as that shown in Fig. 2 (Exp. 3) and is based upon a single determination, there being insufficient material for a duplicate. The curve for *l*-galacto-ascorbic acid is the average of the two curves shown in Fig. 2 (Exp. 3). The curve for *d*-galacto-ascorbic acid is the same as that shown in Fig. 2 whilst that for *d*-gluco-ascorbic acid is the curve which was obtained simultaneously. These curves are thus constructed from data obtained from experiments conducted on consecutive days using the same enzyme preparation.

All the curves shown have been corrected on a basis which assumed the presence of 2·5 mg. of ascorbic acid or its equivalent of analogue. This calculation was carried out from the data of the oxygen uptake at the cessation of the reaction and of the quantity of compound oxidized, which, as was already mentioned, was calculated from the difference between the initial and final titrations.

The composite curves bring out very clearly the points under discussion. In the first place they show that all the compounds with the oxygen ring to the right of the carbon chain are oxidized by the enzyme very much more rapidly than their corresponding enantiomorphs. Secondly, that in the case of the compounds which are more slowly oxidized, namely those in which the ring engages the hydroxyl of carbon atom 4 to the left of the carbon chain, the number of carbon atoms in the molecule also influences the velocity of the dehydrogenation. Whether this holds true for the rapidly oxidizable analogues is impossible to tell from the data available. A greater number of experiments would be required to settle this point. It will receive further attention when more experimental material is available. Another point which emerges from this work is that the reaction in the case of the rapidly oxidizable compounds, unlike that of their corresponding enantiomorphs, proceeds at a linear rate. This fact is brought out more clearly in an experiment graphically represented in Fig. 5 in which the reaction was extended to an hour. During this time, as will be seen from Fig. 4, the oxidations of the slowly oxidizable analogues show a definite retardation in speed. The linear rate of oxidation of the analogues with the oxygen ring to the right of the carbon chain therefore resembles that previously observed in the case of *l*-ascorbic acid by other workers [Szent-Györgyi, 1931; Tauber *et al.* 1935; Hopkins & Morgan, 1936].

Whilst this investigation was in progress a communication [Rosenberg, 1937] appeared in which it was shown titrimetrically that *d*-arabo-ascorbic acid, like *d*-ascorbic acid, was oxidized at a slower rate than *l*-ascorbic acid. This observation with regard to *d*-arabo-ascorbic acid is not in agreement with our results.

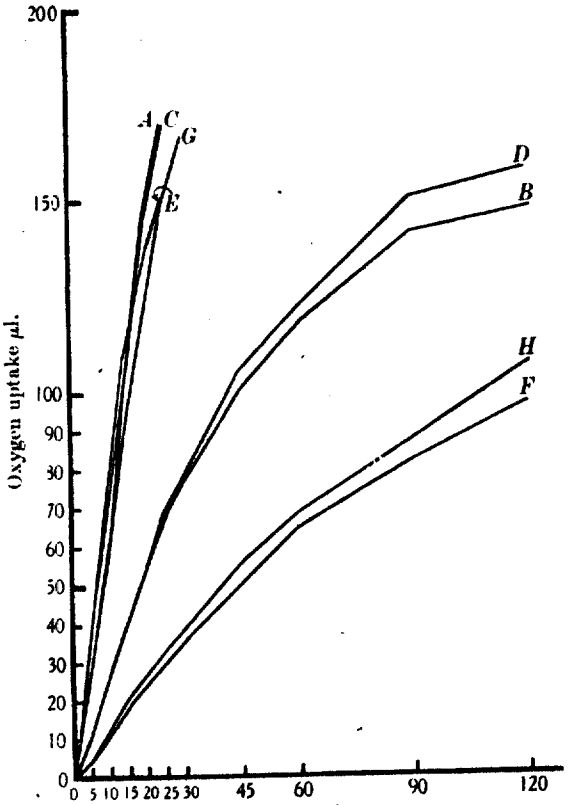


Fig. 4.

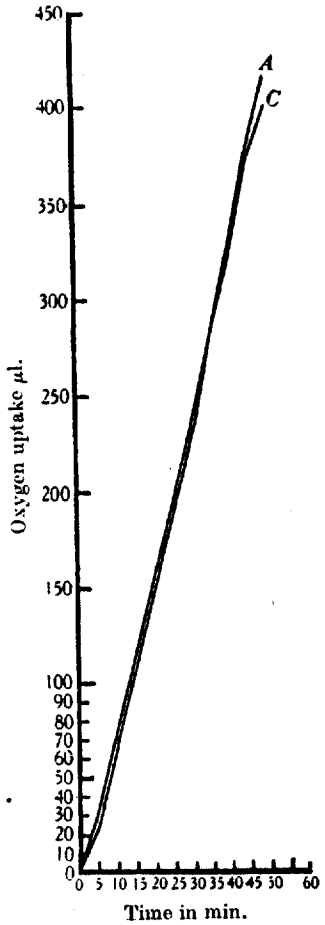


Fig. 5.

Fig. 4. *A*, *l*-ascorbic acid. *B*, *d*-ascorbic acid. *C*, *d*-arabo-ascorbic acid. *D*, *l*-arabo-ascorbic acid. *E*, *l*-gluco-ascorbic acid. *F*, *d*-gluco-ascorbic acid. *G*, *l*-galacto-ascorbic acid. *H*, *d*-galacto-ascorbic acid.

Fig. 5. *A*, *l*-ascorbic acid. *C*, *d*-arabo-ascorbic acid

We should like to point out in this connexion that using different enzyme preparations we have carried out five simultaneous determinations with *d*-arabo-ascorbic acid and *l*-ascorbic acid by the Warburg respirometer method, which we consider a more suitable technique for the purpose in question, and have always obtained identical results. Furthermore, judging from our observations on the dependence of the rates of oxidation of the other analogues upon their stereochemical structure, *d*-arabo-ascorbic acid would be expected to be dehydrogenated

at a velocity similar to that of *l*-ascorbic acid and not of *d*-ascorbic acid as observed by Rosenberg.

Although no ascorbic acid oxidase is present in the animal organism it is of interest, nevertheless, to compare the relationship between the bearing of the stereochemical structure of these compounds on their enzymic oxidation and on their antiscorbutic potency. Of all the compounds so far tested only those with the oxygen ring to the right of the carbon chain have been found to be antiscorbutically active. This was shown to be in all probability due to the fact that the inactive compounds were excreted more freely by the kidney than the active enantiomorphs and that they were not "fixed" by the tissues [Zilva, 1935, 1, 2]. Of the compounds used in this investigation the following failed to show any antiscorbutic activity when administered to guinea-pigs in the doses mentioned, *d*-ascorbic acid 20 mg. [Demole, 1934], *l*-arabo-ascorbic acid 10 mg. [Zilva, unpublished results], *d*-gluco-ascorbic acid 10 mg. [Zilva, 1935, 1], *d*-galacto-ascorbic acid 10 mg. [Zilva, 1935, 1]. They may, therefore, be considered to be inactive. The other compounds were all found to possess some potency. Prof. Reichstein kindly informs me that *l*-galacto-ascorbic acid, for the activity of which there is no record in the literature, has 1/60th of the potency of *l*-ascorbic acid. This compound was also tested in respect to excretion and "fixation" by the guinea-pig and the results again indicated slight activity [Zilva, unpublished results]. There is, therefore, a similarity in so far as the compounds with the oxygen ring to the right of the carbon chain, which are antiscorbutically active, are oxidized by the enzyme more rapidly than the inactive enantiomorphs. This similarity is, however, only partial since *d*-arabo-ascorbic acid possesses only about 1/20th [Dalmer & Moll, 1933; Demole, 1934], *l*-gluco-ascorbic acid about 1/40th [Reichstein, 1934; Zilva, 1935, 1] and *l*-galacto-ascorbic acid 1/60th of the activity of *l*-ascorbic acid [Reichstein, private communication], whilst the rate of enzymic dehydrogenation of this series of compounds is about the same. The asymmetry of carbon atom 5, therefore, conditions the antiscorbutic activity in these six carbon compounds to a marked extent but has no influence upon the kinetics of the enzymic oxidation process. The disparity between the antiscorbutic activity of *l*-ascorbic acid and its seven-carbon homologues is even greater but in this case also no significant difference between their rates of oxidation was recorded.

The results so far obtained thus suggest that the asymmetric carbon atoms outside the ring also condition the antiscorbutic activity but not the rate of the direct enzymic oxidation of *l*-ascorbic acid and its six carbon atom analogues. Whether the rate of the indirect oxidation by enzymes such as the phenolases of the apple or of the potato bears the same relationship to the stereochemical structure of these compounds cannot at present be definitely answered. Preliminary experiments have so far yielded results which are not easily interpreted but the problem is being further investigated.

Summary

Compounds of the ascorbic acid series in which the oxygen ring engages a hydroxyl group to the right of the carbon chain (*l*-ascorbic acid, *d*-arabo-ascorbic acid, *l*-gluco-ascorbic acid and *l*-galacto-ascorbic acid) are directly oxidized by the cucumber ascorbic acid oxidase at a much higher rate than are their enantiomorphs (*d*-ascorbic acid, *l*-arabo-ascorbic acid, *d*-gluco-ascorbic acid and *d*-galacto-ascorbic acid). In the first group of compounds the oxidation proceeds at a linear rate whilst in the second group the rate of dehydrogenation

falls off after a time. In the six-carbon atom compounds of the slowly oxidizable group (*d*-ascorbic acid and *l*-arabo-ascorbic acid) the oxidation proceeds more quickly than in the seven-carbon atom compounds of the same group (*d*-gluco-ascorbic acid and *d*-galacto-ascorbic acid). The relationship of the stereochemical structure of these substances to their enzymic oxidation on the one hand, and their antiscorbutic potency on the other, is discussed.

We should like to express our deep gratitude to Profs. W. N. Haworth and E. L. Hirst for their valuable gifts of *d*-ascorbic acid, *d*- and *l*-arabo-ascorbic acids, *d*- and *l*-gluco-ascorbic acids and *d*-galacto-ascorbic acid and to Prof. T. Reichstein for *l*-galacto-ascorbic acid. *l*-Ascorbic acid was kindly supplied by Messrs Hoffmann-La Roche, Ltd.

One of us (S. W. J.) is indebted to the Medical Research Council for a whole time grant.

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Synthesis of L-Ascorbic Acid in Plants and Animals

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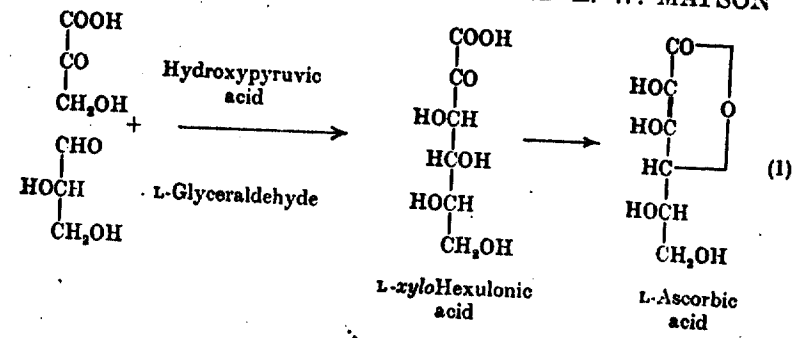
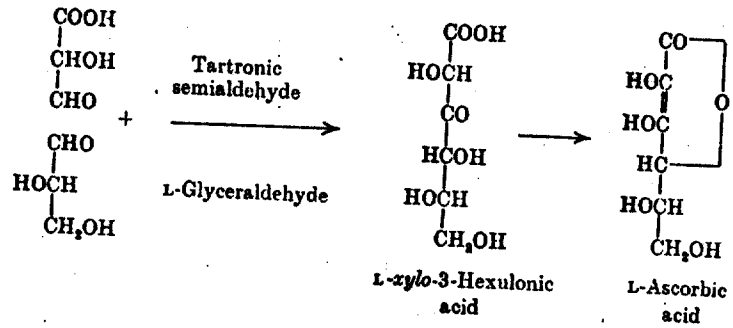
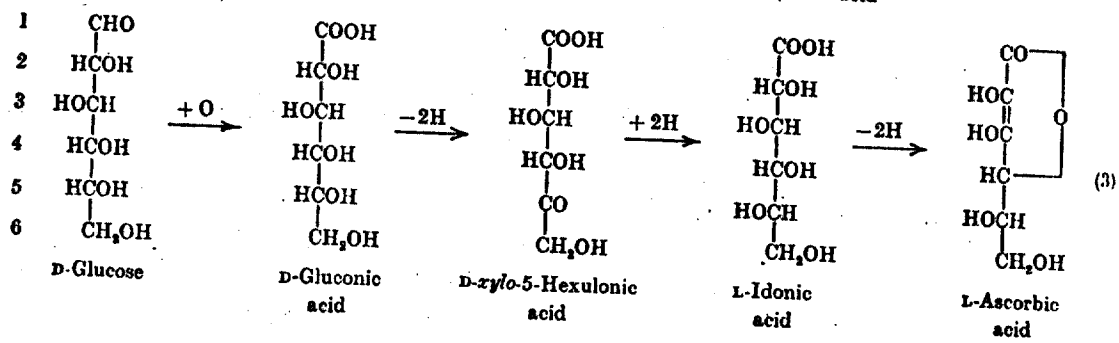
The chemical synthesis of L-ascorbic acid from D-galactose by Haworth & Hirst (1933) forms an outstanding achievement in the chemistry of the carbohydrates, and has frequently stimulated speculation as to whether the biological synthesis of L-ascorbic acid proceeds along similar lines. The very common occurrence of sugars in both plants and animals, and the fact that L-ascorbic acid is closely related to a sugar, L-gulose, has tended to give support to this idea. The idea has not hitherto been supported by any extensive body of facts, although there is a strong suggestion from earlier work that hexose sugars provide the carbon skeleton of the molecule. A number of experiments have been described in the literature which suggest that the carbon skeleton of L-ascorbic acid in both plants (Ray, 1934) and animals (Ruffo & Tartaglione, 1948; Jackel, Mosbach, Burns & King, 1950) is formed from a hexose sugar, though none of them definitely identified any one sugar as the precursor. In the case of plants, some workers (Ray, 1934; Tadokoro & Nisida, 1940; De & Barai, 1949) have claimed that D-mannose is the precursor, whilst others (Sugawara, 1941; Ahmad, Qureshi, Babbar & Sawhney, 1946; Itô & Mizuno, 1948) have suggested that a number of sugars can act as precursors. Such conflicting claims are not unexpected in view of the large variety of plant materials which have been examined. The hexose sugars and probably a number of other compounds such as sorbitol and inositol are interconvertible, but to different extents in different plants, so that the success obtained by feeding any of these compounds may well depend on whether the plant can convert them into the actual precursor of L-ascorbic acid. It is perhaps significant that with every kind of plant material examined D-glucose has caused the formation of L-ascorbic acid, whereas the other sugars, D-mannose, D-galactose and L-sorbose, have in a number of experiments given negative results (Itô & Mizuno, 1948; Devyatnin, 1950; Mapson, Cruickshank & Chen, 1949). In the case of animals the work of Jackel *et al.* (1950) has shown that a direct link exists between D-glucose and L-ascorbic acid though the evidence does not eliminate the possibility that D-glucose may be converted into D-mannose or D-fructose before being transformed into L-ascorbic acid; feeding D-[^{14}C]glucose labelled uniformly in all positions to chloretone-treated rats gave L-ascorbic acid which was also labelled uniformly in all positions. Mosbach and co-workers suggest that the carbon chain of the D-glucose is not broken before being converted to L-ascorbic acid or, if so, the fragments are recombined without any major differential dilution effect. No clue is given in any of the above work as to the series of changes by which the D-glucose is transformed into L-ascorbic acid.

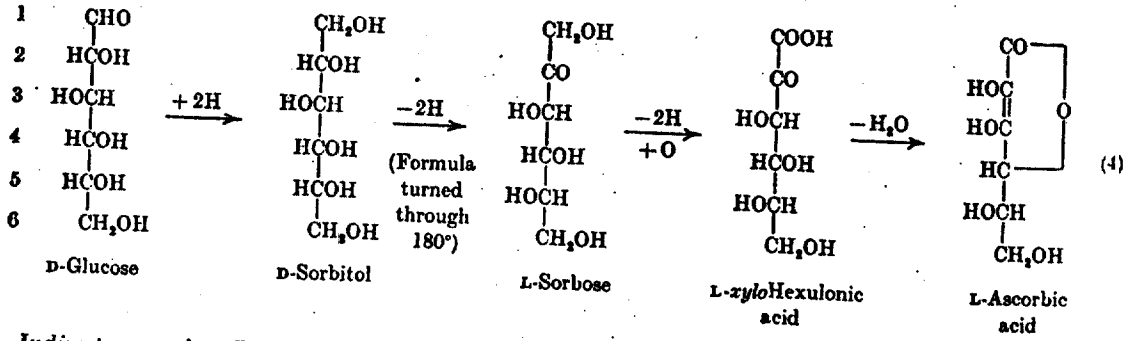
The observation that there is a close link between a hexose sugar, which may be D-glucose, and L-ascorbic acid has stimulated speculation as to the method by which the transformation from one to the other is effected, particularly the means by which a member of the D-series of sugars is changed to a member of the L-series. The investigations to be described in this and subsequent communications are directed towards establishing the nature of the hexose precursor and elucidating the mechanism by which the hexose is transformed into L-ascorbic acid; and the present paper describes evidence in support of a suggested mechanism for the transformation of two common hexoses, D-glucose and D-galactose, into L-ascorbic acid by a series of simple reactions which could reasonably be imagined as occurring in biological systems. Various possible sequences of reactions have been examined in which the main emphasis has been on changes in configuration necessary for the sugar to be transformed into L-ascorbic acid and in which no attempt has been made to portray the sugars or sugar acids in their cyclic forms, even though in practice the sugar acids were fed or injected as lactones in order to facilitate their entry into the cell. In every sequence of reactions the assumption has been made that the starting sugar is D-glucose though, in the brief discussion which follows, an indication has been given as to whether D-galactose or D-mannose could replace D-glucose.

The schemes described represent only a few of the many which on chemical grounds can be suggested for the transformation, but they serve to focus attention on the type of compounds which might be precursors of L-ascorbic acid. For convenience in presentation they have been grouped under two main headings.





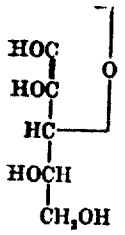




Indirect conversion. Indirect conversion, in which the carbon chain is broken and then reformed, involves the breakdown of the D-glucose via fructose diphosphate to the triose phosphates and the conversion of these into other three-carbon compounds, which then combine to give L-ascorbic acid. Assuming that this occurs, two types of condensation reaction can be suggested.

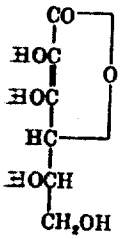
The first (1) is based on an aldol condensation between L-glyceraldehyde and hydroxypyruvic acid (Hough & Jones, 1951, 1952) catalysed by an enzyme similar to the aldolase of animal tissues (Meyerhof, Lohmann & Schuster, 1936) and plant tissues (Stumpf, 1948).

The second type of condensation (2) is an acyloin reaction between tartronic semialdehyde and L-



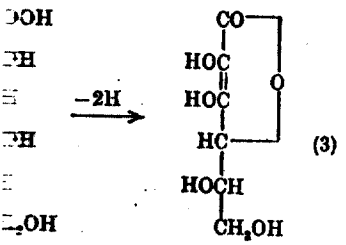
(1)

L-Ascorbic acid



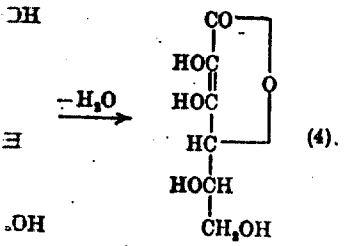
(2)

L-Ascorbic acid.



(3)

L-Ascorbic acid



(4).

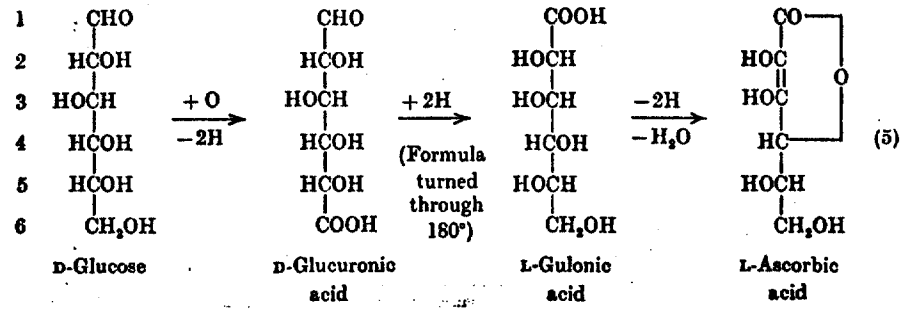
L-Ascorbic acid

based on an aldol condensation hyde and hydroxypyruvic acid , 1952) catalysed by an enzyme se of animal tissues (Meyerhof, er, 1936) and plant tissues

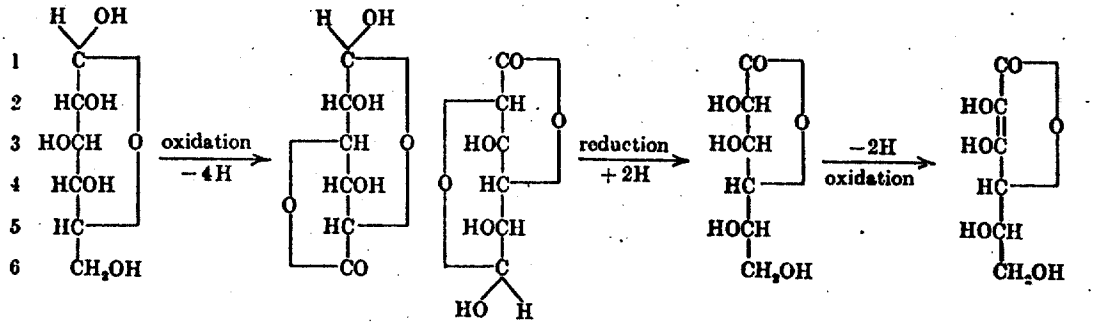
condensation (2) is an acyloin rtronic semialdehyde and L-

glyceraldehyde. L-*xylo*-3-Hexulonic acid (3-keto-L-gulonic acid) is known to be in tautomeric equilibrium with L-ascorbic acid, the equilibrium being very much in favour of the L-ascorbic acid (Cox, Hirst & Reynolds, 1932).

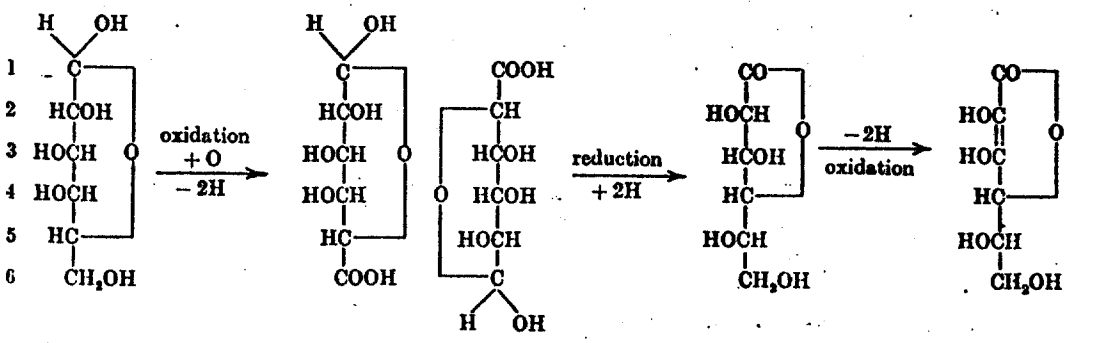
Some support for the general idea of indirect conversion came from the observation, which is described at length in another paper, that the formation of the three-carbon compound D-glyceric acid ran parallel with the formation of L-ascorbic acid in germinating cress seedlings and in rats. However, D-glyceric acid does not appear to be a precursor, for feeding the ethyl esters of D- or DL-glyceric acid to cress seedlings or injecting the compounds into rats did not produce any increase in the synthesis of L-ascorbic acid. It seems possible that the parallel formation of D-glyceric acid and L-ascorbic acid is due to a common cell environment rather than to a direct link between the two compounds and may therefore be misleading.



(5)



(6)



(7)

Direct conversion. The observation of Jackel *et al.* (1950) made it seem possible that the conversion of hexose into ascorbic acid takes place without rupture of the carbon chain. This must involve either the inversion of the configuration of the groups on carbon atom 5, or what amounts to an inversion of all the asymmetric carbons as written by transferring in effect the aldehyde or potential aldehyde group from carbon atom 1 to the terminal atom of the carbon chain. Thus the reduction of $C_{(1)}$ of an aldohexose to —CH_2OH and the oxidation of $C_{(6)}$ to an aldehyde or potential aldehyde would mean that if the formula is now written according to the usual convention (being turned through 180° so as to bring the new aldehyde group at the top) the stereochemical designation of each hydroxyl group (D or L) is necessarily reversed. In the case of D-glucose the hydroxyl group on the new $C_{(5)}$ (old $C_{(2)}$) is now in the correct orientation for L-ascorbic acid.

The inversion of the groups attached to carbon atom 5 could occur through the intermediate formation of a 5-oxo compound, for example as in (3). In such a scheme the configuration of the groups on carbon atoms 2 and 3 is immaterial because in L-ascorbic acid they are not optically active. The configuration of the hydroxyl on carbon atom 4 must be D. This is true of D-mannose but not D-galactose.

The possibility of transferring the potential aldehyde group from carbon atom 1 to the terminal atom of the carbon chain has been thoroughly investigated in the present study. One scheme which illustrates the idea (4) is very similar in outline to the sequence of reactions used for the commercial manufacture of L-ascorbic acid. The configuration of the hydroxyls on carbon atoms 2 and 3 must be D and L, respectively. This is true of D-galactose but not D-mannose.

A further scheme of the same general type is (5). Support for this came from the observation of Smythe & King (1942) that treatment of rats with various drugs (e.g. chloretone) increased the excretion of both D-glucuronic acid (as glucuronide) and L-ascorbic acid. D-Galactose but not D-mannose could replace the D-glucose.

This scheme appears to be fundamentally the correct one. It can be written more conveniently in terms of the derivatives which were actually used to test its applicability to rats and cress seedlings. Scheme 5 for glucose and galactose can then be written as (6) and (7), respectively, which illuminates, in a striking manner, the crucial inversion from the D- to L-configuration by a series of plausible biochemical reactions.

The chief purpose of this paper is to present evidence that the reaction pathways of these last two schemes actually exist in plant and animal tissues. Briefly, the evidence consists of the demonstration that of a large number of closely related sugar derivatives, four sugar acid derivatives, L-gulono-γ-lactone, L-galactono-γ-lactone, D-glucurono-γ-lactone and D-galacturonic acid methyl ester, when fed to germinating cress seedlings or injected into rats, produced a very marked increase in the synthesis of L-ascorbic acid. These compounds are precisely those which should have an effect if the reactions in the above schemes (6) and (7) described the path by which L-ascorbic acid is synthesized; and moreover, no other closely related compound gives rise to L-ascorbic acid.

METHODS

The possibility that the above schemes outline the pathway by which L-ascorbic acid is synthesized from D-glucose has been examined by feeding various compounds postulated as intermediates in schemes 1–5 to cress seedlings, or injecting them into rats, and by comparing the formation of L-ascorbic acid with that in untreated controls. In the case of rats the tissues are normally saturated with L-ascorbic acid so that they excrete in their urine a small approximately constant amount which can be used as a measure of the synthesis of L-ascorbic acid in the intact animal. Any increase in the synthesis of L-ascorbic acid in the tissues is immediately reflected in the urinary excretion. The experiments on cress seedlings and on rats will be described separately.

Before discussing the significance of the results obtained, it is important to emphasize that certain of the compounds fed were simple derivatives of those originally postulated in schemes 1–5. These derivatives were chosen because they would penetrate into the cell, whereas those originally suggested would not do so, and yet once inside the cell would decompose to give the postulated intermediate. In the present study it was considered essential to make sure that the compound had at least penetrated into the seedling itself. Even then, the possibility remained that it might be unable to reach the active centres of synthesis, but for the present study the assumption was accepted that if the compound was present in the sap, it was available for conversion into L-ascorbic acid. Our experience was that organic compounds (Simon & Beevers, 1951) which were ionized in solution penetrated into the cress seedlings little if at all, whereas compounds which were un-ionized in solution, such as esters, lactones and sugars, penetrated readily (Beevers, Goldschmidt & Koffler, 1952). Acids such as galacturonic were not absorbed, but if they were fed as the methyl or ethyl esters, these penetrated into the seedling and then hydrolysed to give the free acid in the sap. α-Hydroxy acids such as galacturonic can be very conveniently handled in this way because their esters usually hydrolyse fairly rapidly in aqueous solution. Sugar acids such as D-gluconic, D-mannonic, L-gulonic and L-galactonic were fed as the γ-lactones. These were chosen because they were internal esters and were therefore free from the criticism that a 'foreign' alcohol was being introduced into the cell which might affect the synthesis of L-ascorbic acid; in aqueous solution they hydrolyse very slowly and might therefore be expected to penetrate into the cell in the same way as the esters.

Preparation of chemicals

Many of the chemicals used in this study were not obtainable commercially and had to be synthesized. Details are given in Table 1.

Each of the compounds described was examined on a paper chromatogram using as solvent either the water-poor phase from a two-phase mixture of *n*-butanol:glacial acetic acid:water (4:1:5 by vol., respectively) or a single phase solvent *n*-propanol plus concentrated ammonia (7:3 by vol. respectively). In the latter solvent the lactones were decomposed and moved as the ammonium salts of the free acids. In all cases except that of L-gluconic acid the compounds were free from other sugar derivatives. Some of the γ-lactones tended to decompose even with the acid solvent to give small amounts of the free acid and the δ-lactone, but these were readily identified.

Analytical technique

Many of the experiments which will be described later depended upon the detection, and in some cases the quantitative estimation, of minor constituents of the cell sap. For some, chemical methods were available, but for many the only method was the examination of the cell sap on a paper chromatogram. A full description of the apparatus and the general conditions of working in order to obtain the maximum resolution of the solutes on the paper chromatogram has been given in an earlier study on the separation of the sugars from plant extracts by Isherwood & Jermyn (1951). In the present paper only the modifications necessary for the separation of the particular compounds under investigation will be described. In many cases the quantitative chromatographic methods supplemented the results obtained by older chemical methods. Analysis of the urine

Table 1. *Preparation and properties of sugar acid derivatives*

(All but one are γ-lactones. Rotations were in water unless specified otherwise and were at room temperature.)

Substance (γ-lactone)	Method of preparation	References	M.p. (°) (uncorr.)	$[\alpha]_D$ (°) (*c* in parentheses)	Notes
D-Allono	From D-ribose (cyanohydrin)	(*a*)	130	−6 (3·5)	—
D-Altrono	From D-ribose (cyanohydrin)	(*a*)	Syrup	+35 (2·7)	Free acid prepared from Ca salt by addition of HCl; $[\alpha]_D$ +8 (4)
D-Gulono	From D-xylose (cyanohydrin)	(*b*)	180	−56 (1)	—
D-Idono	From D-xylose (cyanohydrin)	(*b*) (*c*) (*d*)	Syrup	−53 (10)	Brucine salt of acid, m.p. 182°. Dibenzal derivative, m.p. 204°
D-Mannono	From D-mannose by oxidation	(*e*)	150–152	+51·5 (1)	—
D-Glucono	From D-glucose by oxidation	(*e*) (*f*)	133–136	+68 (5)	—
D-Galactono	From D-galactose by oxidation	(*e*)	132–133	−73 (5)	—
D-Talono	From D-galactonic acid by epimerization	(*g*)	130	−33 (2)	Free acid, m.p. 125°; $[\alpha]_D$ +17 (5)
L-Gulono	From D-glucuronic acid by reduction with Na/Hg	(*h*)	180	+55 (1)	—
L-Idono	From L-gulonic acid by epimerization	(*c*)	Syrup	+50 (5)	Brucine salt of acid, m.p. 190–192; $[\alpha]_D$ −17 (5)
L-Galactono	From D-galacturonic acid by reduction with Na/Hg	(*h*)	132	+75 (5)	—
L-Talono	From L-galactonic acid by epimerization	(*g*)	130	+33 (2)	Free acid, m.p. 125°; $[\alpha]_D$ −17 (5)
L-Mannono	From L-arabinose (cyanohydrin)	(*b*)	150	−51 (5)	—
L-Glucono	From L-arabinose (cyanohydrin)	(*b*)	—	—	Crude syrup after removal of bulk of L-mannono-γ-lactone
D-Mannurono	From alginic acid	(*i*)	138	+92 (3)	—
D-Galacturonic acid methyl ester	From D-galacturonic acid	(*j*)	145	+94 (2, MeOH)	—

References: (*a*) Phelps & Bates (1934); (*b*) Hudson, Hartley & Purves (1934); (*c*) Fischer & Fay (1895); (*d*) Micheel & Kraft (1933); (*e*) Hudson & Isbell (1929); (*f*) Isbell & Frush (1933); (*g*) Glatthaar & Reichstein (1938); (*h*) Thierfelder (1891); (*i*) Frush & Isbell (1946); (*j*) Jansen & Jang (1946).

of the rat was effected by essentially the same methods as those used for the cress seedling.

The analysis consisted mainly in determinations of L-ascorbic acid, sugars and organic acids such as D-glyceric acid.

L-Ascorbic acid was estimated chemically by oxidation with 2:6-dichlorophenol indophenol, the procedure adopted being essentially that described by Harris & Olliver (1942). The quantitative chromatographic method described by Chen, Isherwood & Mapson (1953) will distinguish between D-*arabo*ascorbic acid and L-ascorbic acid and was used particularly in connexion with experiments in which various sugar acid derivatives were fed to cress seedlings or injected into rats.

Fermentable and non-fermentable sugars were estimated by the method of Mapson, Cruickshank & Chen (1949) or by the chromatographic method of Jermyn & Isherwood (1949). The accuracy of the latter method has been much improved by a modification to the copper reagent used for estimating the separated sugar. Wager (private communication) has found that by carrying out the oxidation of the sugar in the absence of oxygen, the precision of the results with small amounts of sugar is very much increased.

In the qualitative examination of the cell sap of the cress seedling, the amount of acid present was judged visually from the size of the spot on the paper chromatogram using solvents recommended by Isherwood & Hanes (1953). This was particularly useful in the examination of cress seedlings which had been fed with various sugar-acid derivatives, when it was essential to know whether the acid had penetrated into the seedling and whether it had noticeably affected any of the other constituents. Further details of the qualitative examination of the cress seedlings will be given later in the description of the separate experiments.

Culture of cress seedlings

The procedure used varied slightly as the work progressed but the preferred method was as follows.

About 300 seeds were placed on a piece of fine Nylon fabric (area about 60 cm.^{2}) which covered the bottom of a white porcelain jar. Underneath the Nylon fabric was a piece of filter paper of about the same size. Sufficient of the appropriate solution was then added so that the seeds were just wetted. Nylon fabric was chosen because it is not absorptive, and if necessary it can be lifted out of the jar with the seedlin[illegible] still in position, fresh solution and filter paper intro[illegible] and the Nylon replaced in its original position. This [illegible]cularly useful when the aqueous solution slowly decomposes as with the sugar acid lactones and esters and it is essential to feed the seedlings with fresh solution each day. The seeds were germinated in the dark at a temperature of 20° and the jars were covered to prevent evaporation.

For analysis the seedlings were usually washed to remove any adherent solution and then the testas removed. The subsequent treatment depended upon whether they were to be analysed for L-ascorbic acid or whether the constituents of the cell sap were to be examined on a paper chromatogram. For the latter they were frozen at −20° and held at this temperature until required.

Treatment of rats with various compounds

Piebald male rats of varying ages but usually about 140 g. in weight were given a basal diet containing casein 20, sucrose 60, arachis oil 15, and salt mixture 5%, with an additional 10% of dried yeast. Each animal received weekly supplements of vitamins A and D by the administration of two drops of halibut-liver oil (= 1500 i.u. of A and D/rat/week, respectively). Vitamin E was given as 1 mg. of (±)-α-tocopherol acetate. The rats were housed in metabolism cages in which all metal parts coming into contact with urine were waxed. The urine was collected in beakers containing 10 ml. of 10% (w/v) oxalic acid to avoid any decomposition of the L-ascorbic acid. The rats were rested until their urinary excretion of L-ascorbic acid was steady and then they were injected subcutaneously with 0·5 ml. of an aqueous solution of the compound to be tested. Commonly 100 mg. of the organic compound was injected.

RESULTS

Experiments with cress seedlings. The effect on the synthesis of L-ascorbic acid of feeding various sugar derivatives

In the experiments described in Table 2, compounds which were postulated as intermediates in various possible sequences of reactions linking glucose with L-ascorbic acid were fed to cress seedlings and the formation of L-ascorbic acid in these seedlings compared with that in seedlings grown in water under identical conditions of temperature and humidity; a fresh control experiment on seedlings grown in water was made for each compound.

The results described in Table 2 show that feeding the γ-lactone of D-gluconic acid (scheme 3), L-sorbose and D-sorbitol (scheme 4) and DL-glyceraldehyde (schemes 1 and 2) did not cause any marked increase in the synthesis of L-ascorbic acid as compared with that in seedlings grown in water. Experiments with D-xylose and L-arabinose were included to show the results obtained when compounds were fed which were neither metabolized by the seedlings to any extent, nor likely to be precursors of L-ascorbic acid. These experiments formed a very useful standard by which to judge the other experiments. Thus it would appear that the synthesis of L-ascorbic acid does not proceed through any of the compounds mentioned above, though it must be emphasized that if phosphorylated derivatives of the compounds were the actual intermediates, then the compounds fed to the seedlings would have to be phosphorylated inside the cell before becoming available for conversion into L-ascorbic acid. This possibility would tend to make any conclusions which might be derived from these feeding experiments rather tentative. However, the striking results obtained by feeding under analogous conditions the γ-lactones of D-glucuronic and L-gulonic acids (scheme 5) diminish the importance of this criticism. These compounds definitely increased the synthesis of L-ascorbic acid even though the cell sap became more acid. The growth and appearance of the seedlings were practically indistinguishable from those grown in water.

Table 2. *Synthesis of* L-*ascorbic acid in cress seedlings grown in solutions of various sugar derivatives*

Sugar derivative	Duration of experiment (hr.)	Weight of 100 seedlings (g.)	L-Ascorbic acid as % of control grown in water	Examination* of cell sap
D-Glucono-γ-lactone (0·5%)	72	1·10	61	pH 4·56, 4·44 C
	96	1·20	43	
	120	1·60	60	
DL-Glyceraldehyde (0·1%)	48	1·0	82	C
	72	1·73	87	
	96	2·51	89	
	120	2·96	95	
	144	3·18	83	
D-Glyceraldehyde (0·1%)	72	1·0	93	C
	96	1·4	108	
	120	1·8	103	
D-Sorbitol (1%)	51	1·03	96	C
	72	1·51	98	
	96	1·88	108	
	120	2·08	102	
	144	2·44	113	
L-Sorbose (1%)	51	1·0	75	U
	72	1·38	88	
	96	1·89	103	
	120	2·14	100	
	144	2·21	103	
D-Glucurono-γ-lactone (1%)	72	1·00	97	C
	92	1·30	125	
	118	1·80	127	
	142	1·90	131	
	163	1·90	149	
L-Gulono-γ-lactone (1%)	72	1·30	152	pH 4·6, 4·59, 4·41 C
	96	1·60	139	
	120	1·80	144	
	142	2·10	137	
D-Xylose (1%)	72	1·0	74	U
	96	—	100	
	140	—	114	
	170	1·90	112	
L-Arabinose (1%)	72	0·96	82	U
	96	—	100	
	140	—	108	
	170	2·35	106	

* U, the cell sap contained an appreciable amount of the sugar derivatives as measured by the rise in the unfermentable sugars. C, examination on a paper chromatogram showed that the cell sap contained an appreciable quantity of the sugar derivative.

Feeding L-*xylo*hexulonic ester also appeared to increase the L-ascorbic acid content but this was later found to be due to the spontaneous conversion of the ester into L-ascorbic acid in aqueous solution, especially in the presence of alkaline buffers (pH 6–8). The L-ascorbic acid was readily absorbed by the seedlings and it thus appears likely that the observed increase was a result of a non-enzymic conversion of the ester into L-ascorbic acid. This experiment, which is not described in Table 2, therefore does not provide unequivocal evidence that the ester is a precursor of L-ascorbic acid in the seedling. In connexion with this discovery, it is interesting to note that it has been claimed that the ester has anti-scorbutic properties (Reichstein & Demole, 1936).

The experiment in which DL-glyceraldehyde was fed deserves closer study. Large concentrations of DL-glyceraldehyde inhibit growth but at the concentration employed in the experiment (0·1%, w/v) described in Table 2, growth was practically unaffected (weight 3·2 g./100 seedlings at 144 days as compared with 2·7–3·4 for seedlings grown in water). The L-ascorbic acid content, however, was definitely lower. This appears to be due to the presence of L-glyceraldehyde, for feeding D-glyceraldehyde in the same concentration did not affect the synthesis of L-ascorbic acid.

The interpretation of feeding experiments with the potentially acid compounds was sometimes complicated by the effect of the compounds on the pH of the cell sap apart from the actual synthesis of

the L-ascorbic acid. An example of this can be quoted from the results in Table 2. D-Glucono-γ-lactone (0·5 %, w/v, in water) fed to cress seedlings clearly depressed the formation of L-ascorbic acid (50 %) because the amount in the sap was sufficient (estimated from paper chromatograms to be about 0·5 % at the sixth day) to make it definitely more acid (pH 4·4) than that of seedlings grown in water (pH 4·8). A rough calculation of the amount by which the synthesis of L-ascorbic is depressed by a change in the pH of the sap can be made from the experiments described by Mapson *et al.* (1949). They showed that the synthesis of L-ascorbic acid in cress seedlings was definitely related to the pH of the sap and was depressed when the sap became more acid. From the figures given in their paper, the change in the pH of the cell sap (4·8–4·4) in the experiment with D-glucono-γ-lactone would be expected to depress the synthesis by about 50 %. The observed depression would therefore appear to be almost entirely related to the change in pH of the cell sap, and it seems unlikely that the compound has any specific inhibitory effect on the synthesis.

The results described in Table 2 suggest strongly that the synthesis of L-ascorbic acid from D-glucose proceeds by a series of reactions similar to those outlined in scheme 5. Later experiments have been concerned with confirming the special position of D-glucurono-γ-lactone and L-gulono-γ-lactone in the synthesis and in establishing the specificity of the various reactions involved.

Examination of the specificity of the reactions involved in the conversion of hexoses to L-ascorbic acid

Cress seedlings. Two reactions outlined in scheme 5 have been examined in detail. Various compounds closely related to D-glucuronic and L-gulonic acids have been fed to cress seedlings and the L-ascorbic acid content compared with that of seedlings grown in water. The results of these experiments have indicated that two hexoses may act as precursors of L-ascorbic acid, namely D-glucose and D-galactose, the sequence of reactions connecting them with L-ascorbic acid being very similar. This was to be expected on theoretical grounds since the configuration of these sugars is such that they can be transferred from the D- to the L-series of sugars by transposing the terminal groups of the molecule as outlined in scheme 5, and the L sugar derivatives produced have the right configuration for a precursor of L-ascorbic acid. This fact was briefly mentioned when scheme 5 was discussed earlier in this paper.

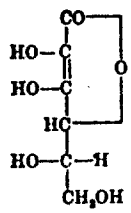
The group of compounds closely related to L-gulonic acid comprised all the known aldohexonic acids. In the feeding experiments, however, the γ-lactones of these were used and the configurations of these, written according to the usual cyclic modification of the Fischer convention, are shown in Fig. 1.

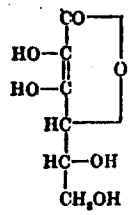
Only two compounds related to D-glucuronic acid (D-glucurono-γ-lactone was actually used) were fed to cress seedlings, namely D-mannurono-γ-lactone and D-galacturonic acid methyl ester. In Fig. 1 these have been drawn in such a way as to emphasize that they have a similar structural relationship to D-mannono- and L-galactono-γ-lactones, respectively, as D-glucurono-γ-lactone has to L-gulono-γ-lactone in scheme 5.

The formulae in Fig. 1 have been grouped so as to bring out as clearly as possible the relationship of the various lactones to L-ascorbic acid. Each horizontal line contains four lactones which have the same configuration as far as the carbon atoms 4 and 5 are concerned but differ between themselves as to the disposition of the groups on carbon atoms 2 and 3. No information can be obtained from the configuration of L-ascorbic acid as to the disposition of the groups on carbon atoms 2 and 3 of its precursor. In Fig. 1 the first line contains lactones which have the same configuration as L-ascorbic acid, the second line lactones in which the configuration of carbon atom 5 is opposite to that of L-ascorbic acid, and the third and fourth lines lactones which are enantiomorphs (mirror images) of the corresponding lactones in the first and second lines respectively.

The results of feeding the various sugar derivatives to cress seedlings are shown in Table 3. It is clear that L-gulono-, L-galactono-, D-altrono-, D-glucurono-γ-lactones and D-galacturonic acid methyl ester markedly increase the formation of an ascorbic-acid-like substance. This is particularly obvious if the results with these sugar derivatives are compared with those obtained with inactive lactones of very similar structure, e.g. D-gulono-, D-galactono- and D-mannurono-γ-lactones. There seems no doubt that all these lactones penetrated into the seedlings (the cell sap was examined on a paper chromatogram) and the difference in behaviour appears to be due to the specificity of the enzymes involved in the conversion to L-ascorbic acid.

The ascorbic-acid-like compound produced by feeding the cress seedlings with L-gulono-, L-galactono- and D-altrono-γ-lactones was separated from the other constituents of the cell sap and examined on a paper chromatogram by the method of Chen *et al.* (1953). Tracings of chromatograms from seedlings which had been fed with L-galactono- and D-altrono-γ-lactones are shown in Fig. 2. (The chromatogram from seedlings fed with L-gulono-γ-lactone has not been included because apart from the very much weaker concentration of the ascorbic-acid-like compound in the sap, the picture was essentially the same as for the L-galactono-γ-


L-Ascorbic acid


D-*arabo*Ascorbic acid

Types I and IV were γ-lactones of aldonic acids, Types V and VI were uronic acid derivatives with the same basic configuration as Types I and II, respectively.

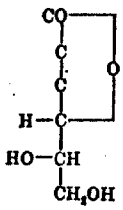
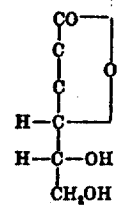
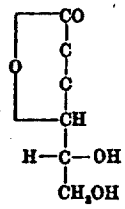
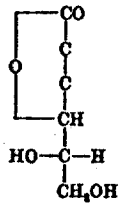
Type (same configuration of groups on $C_{(4)}$ and $C_{(5)}$)		Configuration of groups on $C_{(2)}$ and $C_{(3)}$: HO—C—H / HO—C—H	H—C—OH / HO—C—H	H—C—OH / H—C—OH	HO—C—H / H—C—OH
I		L-GULONO*	L-Idono	L-Talono	L-GALACTONO*
II		D-*Mannono** (rat)	D-Glucono	D-Allono	D-*Altrono** (cress)
III		D-Gulono	D-Idono	D-Talono	D-Galactono
IV		L-Mannono	L-Glucono	L-Allono (not examined)	L-Altrono
V	As I but with —CHO at $C_{(6)}$ instead of —CH_2OH	D-GLUCURONO-* γ-LACTONE	—	—	D-GALACTURONIC ACID METHYL ESTER
VI	As II but with —CHO at $C_{(6)}$ instead of —CH_2OH	D-Mannurono-γ-lactone	—	—	—

Fig. 1. Sugar acid derivatives fed to cress seedlings.

* Compounds in capital letters give L-ascorbic acid, whereas compounds in *italics* give D-*arabo*ascorbic acid, in the cress seedling and in the rat as shown.

Table 3. *Effect of feeding various sugar acid derivatives to cress seedlings on the formation of* L-*ascorbic acid*

(All solutions were put in after 48 hr. germination in water and changed every day. At the time of the first ascorbic acid determination the cell sap was, in every case, examined for the substance fed.)

Sugar derivative fed to seedlings*	Duration of experiment (hr.)	Apparent ascorbic acid as % of that in seedlings grown in water
D-Glucuronolactone	72	97
	96	105
	120	117
	144	123
D-Galacturonic acid methyl ester	72	144
	96	140
	140	172
	164	171
D-Mannuronolactone	96	72
	120	58
	148	57
L-Gulonolactone	72	95
	96	137
	120	146
	144	148
L-Idonolactone	72	103
	96	62
	120	46
L-Talonolactone	72	83
	96	84
	120	69
	144	75
L-Galactonolactone	72	320
	96	256
	120	357
	144	482
D-Mannonolactone	72	102
	96	85
	120	77
	144	79
D-Gluconolactone	72	59
	96	47
	120	55
D-Allonolactone	72	97
	96	77
	120	78
	144	61
D-Altronolactone	72	387 †
	96	552 †
	120	580 †
D-Gulonolactone	72	70
	96	34
	120	27
D-Idonolactone	72	97
	96	56
	120	57
D-Talonolactone	72	127
	96	92
	120	78
	144	77
D-Galactonolactone	72	55
	96	60
L-Mannonolactone	72	76
	96	41
	120	43
L-Gluconolactone	96	64

* All 0·5% (w/v) except D-glucurono- and L-gulono-lactones (1%).

† D-*arabo*Ascorbic acid produced.

lactone). Comparison of these chromatograms indicated that feeding L-galactono-γ-lactone increased the L-ascorbic acid content of the seedlings, and feeding D-altrono-γ-lactone increased the D-*arabo*ascorbic acid content.

The identification of these compounds as L-ascorbic acid and D-*arabo*ascorbic acid is admittedly not conclusive because the enantiomorphic forms would behave similarly on the chromatogram. However, if the configurations of the lactones which act as precursors in the cress seedling are taken into consideration, this uncertainty is removed; L-gulono- and L-galactono-γ-lactones can produce only L-ascorbic acid, and D-altrono-γ-lactone, D-*arabo*ascorbic acid.

Before discussing the results with cress seedlings, the comparable experiments with rats will be described.

Rats. The results of a number of the experiments on rats are shown in Fig. 3. Most of the experiments in which the titration figure for urine remained unchanged have not been described. The same series of sugar-acid γ-lactones were examined as in the case of cress seedlings.

From Fig. 3 it is clear that L-gulono-, L-galactono, D-mannono and D-glucurono-γ-lactones and D-galacturonic acid methyl ester increase the titration figure. Examination of the urine showed that D-mannono-γ-lactone gave rise to D-*arabo*ascorbic acid, whereas the others all gave L-ascorbic acid. The chromatograms were very similar to those shown previously in Fig. 2 for cress seedlings. It was interesting to note that neither D-altrono-γ-lactone, which might give D-*arabo*ascorbic acid, nor L-idono- and L-talono-γ-lactones, which are very similar in structure to L-gulono- and L-galactono-γ-lactones, respectively, produced any increase in the titration figure. D-Mannurono-γ-lactone which by analogy with D-glucurono-γ-lactone in scheme 3 should give D-mannono-γ-lactone on reduction

does not give D-*arabo*ascorbic acid. D-Mannono-γ-lactone has been shown previously to give D-*arabo*ascorbic acid. It seems that the enzyme system which converts the uronic acid to the corresponding aldonic acid cannot convert D-mannurono-γ-lactone into D-mannono-γ-lactone.

The four sugar-acid derivatives which have been shown to act as precursors of L-ascorbic acid in cress seedlings and rats can be regarded as intermediates in two hypothetical series of reactions linking the D-hexoses with L-ascorbic acid. The first of these links D-glucose, D-glucuronic acid and L-gulonic acid to L-ascorbic acid and the second, which is very similar to the first, links D-galactose, D-galacturonic acid and L-galactonic acid to L-ascorbic acid. The only difference between the intermediates in the two series of reactions is the disposition of the groups on carbon atom 4 (carbon atom 3 if based on L-ascorbic acid), those in the D-glucose series having the hydroxyl on the right-hand side (formula written with the aldehyde group at the top) and those in the D-galactose series having it on the left. Although the experimental evidence did not allow a decision to be made as to whether D-glucose or D-galactose was the precursor, it was clear that the enzymes catalysing the series of reactions in the animal were not exactly the same in the rat as those in the plant, because their behaviour towards lactones which might form D-*arabo*ascorbic acid was markedly different. Feeding D-altrono-γ-lactone to cress seedlings gave D-*arabo*ascorbic acid but injected into rats had no effect, whereas feeding D-mannono-γ-lactone to cress seedlings had no effect but injected into rats gave D-*arabo*ascorbic acid. A possible explanation for these observations is based on the similarity in structure of L-galactono- and D-altrono-γ-lactones, and L-gulono- and D-mannono-γ-lactones, respectively (see Fig. 1). Each pair of lactones has the same ring structure and differs only in the disposition of the groups in carbon atom 5. It would appear that the enzyme in cress seedlings which converts either L-gulono- or L-galactono-γ-lactones into L-ascorbic acid, reacts more readily with the L-galactono-γ-lactone ring structure, whereas the corresponding enzyme in the rat reacts more readily with the L-gulono-γ-lactone ring structure. This specificity is only shown when an 'unnatural' lactone is present as substrate.

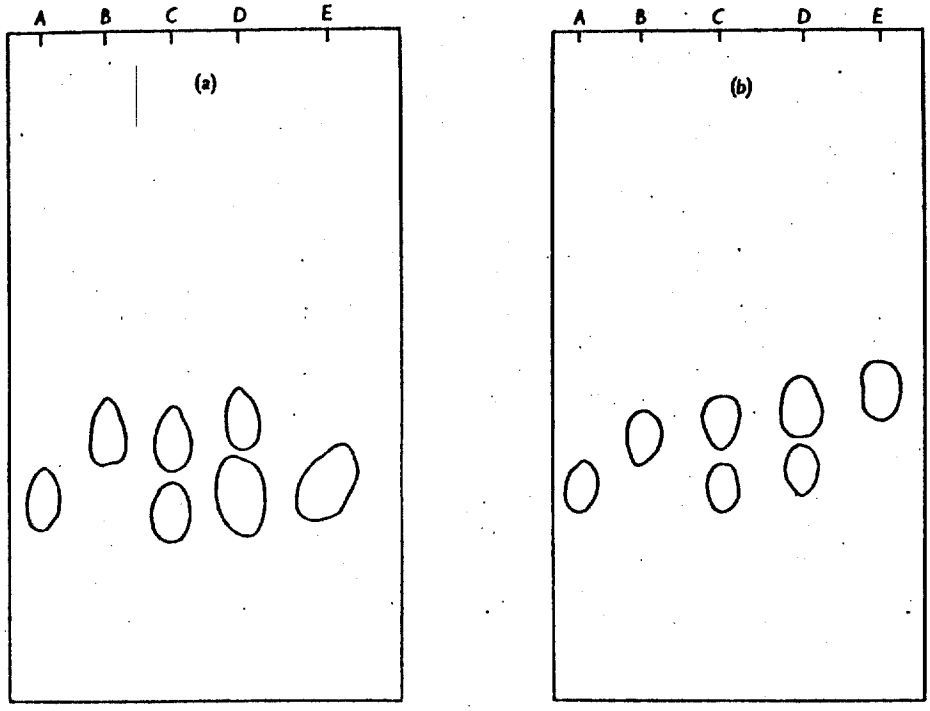


Fig. 2. Tracings of chromatograms showing the compounds present in the sap of cress seedlings which reduce 2:6-dichlorophenol indophenol. The cress seedlings were cultured either in a 0·5% (w/v) aqueous solution of D-altrono-γ-lactone (*a*), or in a 0·5% (w/v) solution of L-galactono-γ-lactone (*b*), for 5 days. Paper, Whatman no. 1. Solvent, phenol:water:oxalic acid. For comparison solutions (0·2%, w/v) of D-*arabo*ascorbic and L-ascorbic acids were spotted on the 'starting line' as shown. *A*, D-*arabo*ascorbic acid; *B*, L-ascorbic acid; *C*, L-ascorbic and D-*arabo*ascorbic acids; *D*, extract of seedlings, L-ascorbic and D-*arabo*ascorbic acids; *E*, extract of seedlings.

The substrate specificity of the enzyme catalysing the conversion of the aldonic acid γ-lactones to ascorbic-acid-like compounds deserves mention, because of sixteen possible precursors only four were active as substrates, two giving L-ascorbic acid. From the results described in Table 3 it is clear that the enzyme requires the disposition of groups on carbon atom 2 to be such that the hydroxyl is on the left-hand side of the molecule. L-Idono- and L-talono-γ-lactones, which are very similar to L-gulono- and L-galactono-γ-lactones, respectively, were without activity. These first two lactones have the hydroxyl on carbon atom 2 on the right-hand side.

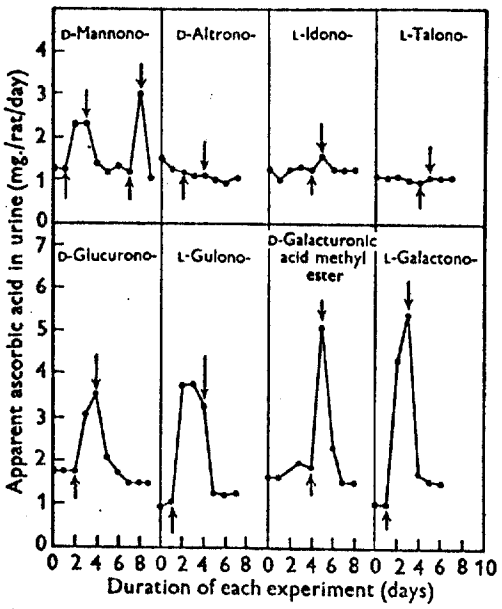


Fig. 3. Effect of injecting rats with various sugar acid derivatives on the excretion of ascorbic acid in the urine. The compounds were γ-lactones in all but one case. ↑ Marks the day the rats were first injected. ↓ Marks the day after which the rats were no longer injected.

The disposition of the groups on carbon atom 3 is not so important for both L-gulono and L-galactono-γ-lactones are active. In these the disposition of the groups on carbon atom 3 is opposite.

It seems essential for the ring to be on the right-hand side of the molecule (hydroxyl on carbon atom 4 on right-hand side), for only lactones which had the ring on the right-hand side were active as substrates.

The efficiency of the conversion of the aldonic acid lactone to L-ascorbic acid is difficult to estimate because it is probable that in the rat a high proportion of the lactone is excreted unchanged and in the cress seedling is converted to the free acid. L-Ascorbic acid (100 mg.) injected into the rat was excreted to the extent of about 60% and by analogy it is possible that a similar proportion of the sugar-acid lactones may be excreted unchanged. The observed ratio between the L-ascorbic acid in the urine and the lactone injected was approximately 3:100 in most of the experiments, though in one experiment when L-galactono-γ-lactone was injected as an aqueous suspension, the ratio was higher, 8:100. This suggests that the efficiency of the conversion might be higher if the lactone was fed continuously so as to maintain a low concentration in the blood, rather than injected all at once. In the case of cress seedlings the efficiency of the conversion has been estimated very roughly to be of the same order of magnitude. The main source of error lies in the fact that the amount of the lactone in the cell sap has been estimated visually from the size of the spot on a paper chromatogram.

CONCLUSIONS

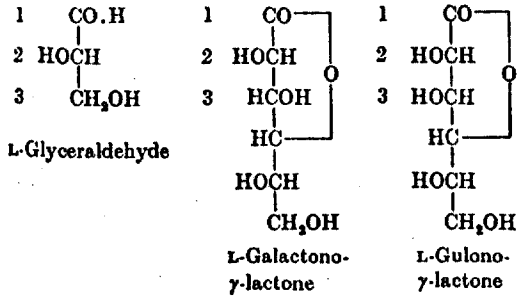
The foregoing experiments constitute a preliminary survey of the series of reactions which convert a hexose, which may be D-glucose or D-galactose, into L-ascorbic acid in both cress seedlings and the rat. Four compounds, L-gulono-, L-galactono- and D-glucurono-γ-lactones and D-galacturonic acid methyl ester, when fed to cress seedlings or injected into rats, have been shown to be transformed into L-ascorbic acid. The significance of this observation is very much increased by the fact that the other sugar-acid derivatives with closely related configurations such as L-idono- and L-talono-γ-lactones are inactive, except that two lactones, D-mannono-γ-lactone in the rat and D-altrono-γ-lactone in the cress seedling, are converted into D-*arabo*ascorbic acid. The four compounds described above have previously in this paper been postulated as intermediates in a hypothetical series of reactions (scheme 5) linking D-glucose (and D-galactose) with L-ascorbic acid, and the fact that these compounds can be transformed into L-ascorbic acid lends considerable support to this hypothesis.

It is possible to explain the conversion of these compounds into L-ascorbic acid by a series of reactions other than those described in scheme 5. In scheme 5 the carbon chain of the hexose was not broken but it is easy to imagine a series of reactions in which the carbon chain was broken down to C_2, C_3 or C_4 fragments and then resynthesized to give L-ascorbic acid. The experiments in the present study do not provide a rigid proof that the carbon chain of the hexose molecule remains unbroken but the earlier observations of Jackel *et al.* (1950) that feeding D-glucose uniformly labelled with ^{14}C to chloretone-treated rats gave L-ascorbic acid which was also uniformly labelled, also suggested that the carbon chain was not broken.

A critical test would be to feed D-glucose which was labelled only in one position along the carbon chain (carbon atom 1 or 6 would be convenient) to rats and then examine the distribution of the labelled carbon along the carbon chain of the resultant L-ascorbic acid. If the chain remained unbroken, the labelled carbon would only occur in the corresponding position along the carbon chain of the L-ascorbic acid. Recently two experiments of this type have been described, the results of which strongly support the reaction chain described in scheme 5. Administration of D-[1-^{14}C]glucose to chloretone-stimulated rats resulted in the urinary excretion of ascorbic acid containing ^{14}C chiefly in position 6 (Horowitz, Doerschuk & King, 1952). Similarly, administration of D-[6-^{14}C]glucose gave ascorbic acid containing ^{14}C in position 1 (Horowitz & King, 1953). The D-glucose must have been converted to ascorbic acid without breaking the carbon chain and a complete inversion of the configuration must have occurred.

In the present study two series of reactions (schemes 1 and 2) based on the condensation of L-glyceraldehyde with appropriate three-carbon compounds to give L-ascorbic acid were tested by feeding DL- and D-glyceraldehyde to cress seedlings. It was found that the formation of L-ascorbic acid was unaffected by feeding D-glyceraldehyde but was definitely depressed by the DL-glyceraldehyde. From these results it seemed unlikely that L-glyceraldehyde was concerned in the synthesis of L-ascorbic acid. The reduced formation of L-ascorbic acid by feeding DL-glyceraldehyde was interesting because L-glyceraldehyde (L-sorbose 1-phosphate is the active inhibitor, Lardy, Wiebelhaus & Mann, 1950) is known to inhibit the hexokinase enzyme present in animals and plants which catalyses the formation of D-glucose 6-phosphate from D-glucose.

If this were true of the hexokinase present in cress it would suggest that glucose 6-phosphate is concerned in the chain of reactions from D-glucose to L-ascorbic acid. However, another explanation of the inhibition can be suggested based on the similarity of the configuration of L-glyceraldehyde and the first three carbon atoms of L-gulono- and L-galactono-γ-lactones.



The resemblance is even closer than the formulae suggest because the carbonyl group at C-1 of the lactones has characteristic carbonyl properties which are absent when it exists as part of a carboxyl ion; presumably resonance in the group $C\begin{matrix}O\\O\end{matrix}\}H$ masks the true carbonyl properties in this case. The supposition is that the L-glyceraldehyde having a similar structure competes with the γ-lactones for the enzyme system which catalyses their oxidation to L-ascorbic acid.

The specificity of the enzymes catalysing the transformation of an aldonic acid to L-ascorbic acid requires that the hydroxyls on carbon atoms 2 and 5 (compared to L-ascorbic acid) shall have the L configuration, and that on carbon atom 4 the D configuration. An additional degree of specificity is implied by the fact that D-altrono-γ-lactone only in cress seedlings and D-mannono-γ-lactone only in the rat are converted into D-*arabo*ascorbic acid. This would suggest that the enzyme in the cress seedling reacts only with compounds which have a configuration for the first four carbon atoms similar to L-galactonic acid (D-altronic acid), whereas the enzyme in the rat reacts only with compounds similar to L-gulonic acid (D-mannonic acid). In the case of L-gulonic and L-galactonic acids, which as lactones give rise to L-ascorbic acid in both the rat and cress seedlings, it is conceivable that the two compounds can be converted into each other as in the enzymic conversion of D-galactose 1-phosphate to D-glucose 1-phosphate by a direct inversion of the groups at carbon atom 4 (Leloir, 1951; Topper & Stetten, 1951). The configuration of L-gulose and L-galactose closely corresponds to that of D-glucose and D-galactose, respectively (cf. scheme 5).

The specificity of the enzymes which catalyse the conversion of a uronic acid presumably via the related aldonic acid (scheme 5) to an ascorbic acid-like compound, has been tested in one case only. D-Mannuronic acid-γ-lactone on reduction gives D-mannonic acid, which (as the lactone) had earlier been shown to give D-*arabo*ascorbic acid in the rat. Injection of D-mannurono-γ-lactone into the rat did not affect the 2:6-dichlorophenol indophenol titration figure, so that it appeared that the enzyme was incapable of reducing D-mannuronic acid to D-mannonic acid. This is in marked contrast to D-glucurono-γ-lactone and D-galacturonic acid methyl ester which definitely are converted into L-ascorbic acid. The only difference between D-mannuronic acid and D-glucuronic acid is in the configuration of the groups on carbon atom 2.

The discovery that the conversion of D-glucose to L-ascorbic acid probably proceeds along a pathway similar to that described in scheme 5 raises the important question as to what difference there is between animals such as guinea pigs and man,

which can suffer from scurvy and are incapable of synthesizing L-ascorbic acid, and those, such as the rat, which do not suffer from scurvy. It appears that in all the animals the synthesis can proceed as far as D-glucuronic acid; even in man, normal urine contains 150–200 mg. of D-glucuronic acid per day, and when compounds such as menthol are administered the excretion of conjugated glucuronides is increased many times. The obstacle to the conversion of D-glucose into L-ascorbic acid would therefore appear to lie between D-glucuronic acid and L-ascorbic acid. In a later paper it is hoped to consider this question in more detail.

The discovery of a series of reactions which describe in broad outline the pathway by which the hexose sugars are converted into L-ascorbic acid is important because it opens up the subject for rapid advances on the enzymic side, though it is possible that the reactions involved in the transformation may not be so simple as in the above scheme. The experimental evidence does not allow us to determine the exact ring structure of the sugar derivatives which are the precursors of L-ascorbic acid, for in the solution in the cell fluids several different forms of the sugar derivatives will be present. In addition, no attention has yet been given to phosphorylated derivatives because at present there is no experimental evidence which definitely indicates that phosphate is concerned, but the intermediates in the transformation may well be phosphorylated analogues of the sugar acid compounds described above.

The fact that the ring structure of the γ-lactones of L-gulonic and L-galactonic acids is similar to that of L-ascorbic acid suggests that the intermediate may be a γ-lactone. However, the ready conversion of the methyl ester of D-galacturonic acid (no lactone ring is present) into L-ascorbic acid and the fact that the γ-lactones are formed very slowly at room temperature in the absence of catalysts suggests that the active intermediate may be a derivative of the sugar acid other than the lactone.

SUMMARY

1. Four compounds, L-gulono-, L-galactono- and D-glucurono-γ-lactones and D-galacturonic acid methyl ester, when fed to cress seedlings or injected into rats, have been shown to be transformed into L-ascorbic acid. A number of other sugar acid lactones with closely related configurations such as L-idono and L-talono-γ-lactones are inactive, except that two lactones, D-mannono-γ-lactone in the rat and D-altrono-γ-lactone in the cress seedling, are converted into D-*arabo*ascorbic acid.

2. The four compounds described above have been postulated as intermediates in a hypothetical series of reactions linking D-glucose (and in a similar series D-galactose) with L-ascorbic acid. The series of reactions in outline are:

D-glucose → D-glucuronic acid → L-gulonic acid → L-ascorbic acid

and

D-galactose → D-galacturonic acid → L-galactonic acid → L-ascorbic acid.

3. The specificity of the enzymes catalysing the transformation of the aldonic acid to L-ascorbic acid requires that the hydroxyls on carbon atoms 2 and 5 (compared to L-ascorbic acid) shall have the L-configuration and that on carbon atom 4 the D-configuration. An additional degree of specificity is implied by the fact that D-altrono-γ-lactone in cress seedlings and D-mannono-γ-lactone in the rat are converted into D-*arabo*ascorbic acid. This would suggest that the enzyme in cress seedlings will react only with compounds which have a configuration for the first four carbon atoms similar to L-galactonic acid (D-altronic acid) whereas the enzyme in the rat will react only with those which have a configuration similar to L-gulonic acid (D-mannonic acid).

4. A survey of a number of possible mechanisms by which the hexoses might be transformed into L-ascorbic acid has shown that those which involve L-glyceraldehyde, L-sorbose, D-sorbitol and D-gluconic acid are not present in cress seedlings or the rat.

We are indebted to Dr Dorothy M. Needham, F.R.S., of the School of Biochemistry, Cambridge, for the gift of a sample of D-glyceraldehyde. Mr F. C. Barrett has assisted with many of the preparations of the sugar lactones and the chromatographic isolation of D-glyceric acid. The work described in this communication forms part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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Isolation of D-Glyceric Acid from Cress Seedlings and its Relationship to the Synthesis of L-Ascorbic Acid

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In the preceding paper (Isherwood, Chen & Mapson, 1953) it has been shown that the transformation in cress seedlings and rats of the two common hexoses D-glucose and D-galactose into L-ascorbic acid can be represented in outline by the following series of reactions:

D-glucose → D-glucurono-γ-lactone → L-gulono-γ-lactone → L-ascorbic acid

and

D-galactose → D-galacturonic acid methyl ester → L-galactono-γ-lactone → L-ascorbic acid

The possibility that the transformation might occur as the result of the breakdown of the hexose into two three-carbon compounds and that the synthesis of L-ascorbic acid might start from these was also considered but was rejected in favour of the scheme given above in which the carbon chain of the hexose is not broken.

The present paper describes for the first time the isolation of the three-carbon compound, D-glyceric acid, from cress seedlings and examines the relationship between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid during the growth of the seedlings. D-Glyceric acid was shown not to be a precursor of L-ascorbic acid though its synthesis is in some way coupled with that of L-ascorbic acid.

EXPERIMENTAL

Methods

Culture of cress seedlings. This has been described previously (Isherwood *et al.* 1953). In most of the preliminary experiments the seedlings were grown in 0·02 M-$(NH_4)_2SO_4$ or water or 0·04 M-$NaHCO_3$ for 96 hr. It was found that the amounts of L-ascorbic acid present at this time in each group of seedlings were approximately 0·2, 0·6 and 0·9 mg./100 seedlings, respectively. After 96 hr. the rate of formation of L-ascorbic acid began to decline. If the seedlings are grown for shorter periods, the rate of L-ascorbic acid synthesis may be somewhat higher, but the seedlings are much smaller and it is difficult to separate the testas from them.

Estimation of L-ascorbic acid and sugars. The methods used have been described previously (Isherwood *et al.* 1953).

Chromatography. In preliminary experiments an extract of germinating cress seedlings, prepared by grinding them with an equal weight of ethanol and centrifuging, was examined in a paper chromatogram using either *n*-butanol:acetic acid:water (4:1:5 by vol. respectively) or

THE EFFECTS OF ERYTHORBIC ACID TREATMENTS ON MELANIN SYNTHESIS AND ON THE ADULT LIFE SPAN IN DROSOPHILA MELANOGASTER: PRELIMINARY REPORT*

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Communicated by Theodosius Dobzhansky, October 7, 1966

Earlier studies had shown that the accumulation of pigment on the abdominal sternites of *Drosophila* was an age-related, genetically controlled phenomenon.[1-3] Experiments in which the time of initial appearance of pigment was employed for predicting future differences in the life span of the treated populations have been described elsewhere.[4, 5] In the present communication, the nature of the pigment and the effects of a metabolic inhibitor on pigment synthesis and on the life span of the *Canton* inbred line of *Drosophila melanogaster* will be described.

In order to identify the pigment, *Canton* males and females were collected in pairs in shell vials and were allowed to age. Individuals of known ages were taken out at random and fixed in Kahle's fixative. Alternative Nile blue method[6] was employed for staining the sternal cuticle and whole mounts of sternites were prepared. Sternites were also examined in sections. The specimens were dehydrated and infiltrated by following the procedures of Smith.[7] Five-micron-thick sections were cut and stained by using Masson-Fontana and ferrous iron methods.[6] These methods revealed that the pigment on sternites was largely extracellular melanin (Fig. 1). The dark bands on the tergites were also found to be due to melanin.

The formation of melanin on the sternites was inhibited in individuals fed on the food medium containing erythorbic acid (an epimer of L-ascorbic acid having only 5% of the vitamin C activity of ascorbic acid). The following experiment was designed to study the effects of erythorbic acid treatments on the duration of life. *Canton* individuals were collected and were divided into five groups. Each pair was provided every day with 3 ml of the food medium in a plastic stopper fitted on a shell vial. All populations were given the normal food medium on the emergence day, but thereafter individual groups were treated differently. Although the amount of erythorbic acid added to the normal food medium was kept constant, i.e., 175 mg of erythorbic acid per 3 ml of the food medium, different groups were given erythorbic acid treatment for different periods of time. The first and second groups of males and females were provided, respectively, with the normal food medium and that containing erythorbic acid throughout the adult life; the third group was given the latter food medium on every third day during the entire life span; the fourth group, on every third day until the 20th day; the last group, on every fourth day up to the 34th day of the imaginal life. Life tables were prepared (for the techniques of handling *Drosophila* populations and for preparing life tables, refer to refs. 8 and 9) by recording the number of deaths in each population each day at 25 ± 1.00°C. For convenience, the five groups will be referred to as I, II, III, IV, and V, respectively.

Table 1 shows the average duration of life in the treated and untreated populations. A shorter average life span of the two sexes is observed in the II and III

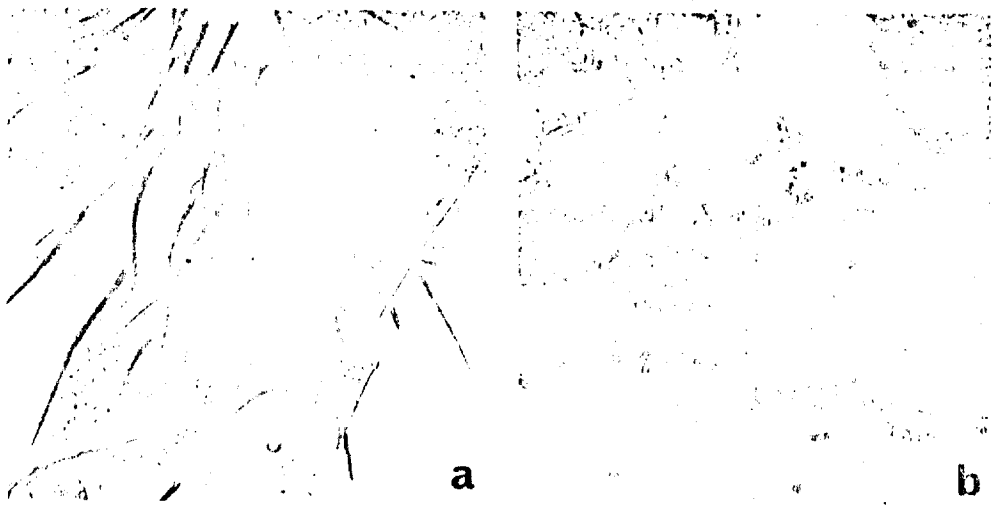

FIG. 1.—Darkly stained melanin pigment on the sternites of 30-day-old *Canton* females of *Drosophila melanogaster*. (*a*) Whole mount of the sternal cuticle (×600); (*b*) transverse section (×2280) passing through a sternite.

groups, although in the latter group only males reveal a significant reduction in longevity compared with the control groups I. In the remaining groups, IV and V, the life span of both sexes is considerably increased in comparison with the untreated population. Individuals in populations with shorter life spans show collapsed abdomens and from nearly absent to very little melanin on the sternites. No such changes are observed in the group III females with unaffected life span. In the long-lived populations, only delay in the time of initial appearance of melanin pigment is observed.

These results suggest that more frequent treatment with erythorbic acid disrupts the normal metabolic processes to such an extent that the expectation of life of the treated groups is reduced. The nature of the metabolic effects of erythorbic acid

TABLE 1

AVERAGE LIFE SPAN IN DAYS IN THE EXPERIMENTAL AND CONTROL POPULATIONS AT 25 ± 1.00°C

Group		No.	Mean life span	Standard error	Coefficient of variability (%)	t	P
I	Males	62	30.08	±1.00	26	—	—
	Females	70	35.70	±0.84	19	—	—
	Sexes combined	132	33.09	±0.69	24	—	—
II	Males	74	8.55	±0.23	23	22.66	<0.001
	Females	74	11.48	±0.24	18	28.15	<0.001
	Sexes combined	148	10.00	±0.20	25	33.46	<0.001
III	Males	70	25.58	±1.08	35	3.04	<0.01
	Females	68	33.75	±1.22	29	1.31	≦0.20
	Sexes combined	138	29.60	±0.88	35	3.11	<0.01
IV	Males	96	32.92	±0.85	25	2.13	<0.05
	Females	97	41.87	±0.68	16	5.71	<0.001
	Sexes combined	193	37.42	±0.62	23	4.55	<0.01
V	Males	92	34.36	±0.94	26	3.03	<0.01
	Females	95	39.77	±1.15	28	2.66	<0.01
	Sexes combined	187	37.11	±0.77	28	3.68	<0.01

The columns t and P compare the treated series with the control.

is yet to be investigated. The increased life span and decreased melanin synthesis in populations which were given erythorbic acid treatment less frequently may perhaps be due to the processes affecting the tyrosine pathways. Since the formation of melanin requires the action of an enzyme, copper-containing tyrosinase, it is likely that the reduction in melanin synthesis may be due to the processes affecting tyrosinase formation or activity. Such a change in tyrosinase could be related to an alteration in the pattern of protein synthesis. The tyrosinase formation and activity, and protein metabolism in the experimental and control populations are under investigation.

Summary.—The pigment on the sternites of the *Canton* inbred line of *Drosophila melanogaster* is identified as extracellular melanin, the formation of which is inhibited by feeding individuals on the food medium containing erythorbic acid. Treatment with erythorbic acid for longer periods results in the inhibition of melanin formation on the sternites, collapsed abdomens, and a reduction in the life span of the treated individuals. The observed results are interpreted as due to drastic changes in the normal metabolic processes. Populations given erythorbic acid treatment for shorter periods reveal a delay in the time of initial appearance of melanin pigment on the sternites and an increase in the life span. These effects are explained as due to the processes modifying the formation or activity of tyrosinase and affecting the pattern of protein synthesis.

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* The experiments were conducted at the Department of Laboratories, Newark Beth Israel Hospital, Newark, N.J., during the period when K. C. S. was the recipient of a Rutgers Research Council faculty fellowship.

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EFFECTS OF ASCORBIC ACIDS AND FLAVONOIDS ON PERMEABILITY OF DERMAL CONNECTIVE TISSUE IN RATS AND GUINEA PIGS (1)

BY

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(2 figures)

Hyaluronic acid is a major component of the ground substance of soft connective tissue. From its tendency to form gels at low concentrations and its ability to complex with proteins, it may control the permeability of the components of interstitial fluid in tissues.

Hyaluronic acid is degraded rapidly *in vitro* by a number of naturally occurring substances of which L-ascorbic acid is one of the most active organic compounds. This reaction has been called the ORD reaction and may be of significance as a process by which hyaluronic acid is degraded *in vivo* (PIGMAN and RIZVI, 1959; PIGMAN, RIZVI and HOLLEY, 1961; FABIANEK, HERP, DAU and PIGMAN, 1961).

In the present work, some substances active in the *in vitro* system were compared for their effect on tissue «permeability» *in vivo*.

A number of methods for measuring tissue « permeability » *in vivo* have been used by earlier workers. Two of these have been modified and used in the present work. One is a modification of the spreading test of DURAN-REYNALS (1928, 1929, 1942; HECHTER, 1947; HECHTER, SHREWSBURY, DOPKEEN and YUDELL, 1947), in which the rate of spreading of Evans blue dye injected in the dermis was measured. The other is a modification of the constant pressure infusion method of LENSTRUP (LENSTRUP, 1951; DAUBENMERKL, 1951 and VENTURI, 1953. This method is based on the rate of subdermal infusion of isotonic saline or phosphate buffer.

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The present studies were made with white rats and guinea pigs. The effects of L-ascorbic acid, D-isoascorbic acid, the oxidized forms of these acids, and several flavonoids were studied. The flavonoids are of special interest since they are reported to increase vascular resistance (ARMENTANO, von BENSÁTH, BÉRES, KUSZNYÁK and SZENT-GYÖRGYI, 1956; FABIANEK, 1957) and influence the amount of ascorbic acid in the organs of guinea pigs (LAVOLLAY and NEUMANN, 1959; FABIANEK, 1961 *a*).

Methods and Materials

The studies of the effects of L-ascorbic acid, D-isoascorbic acid and the dehydroascorbic acids were carried out with rats. Those with flavonoids were carried out with rats and guinea pigs. The animals of both species were 3 to 4 months old, except for some one year old guinea pigs used in the flavonoid experiments. The rats were fed *ad lib.* on Wayne Lab Blox (mice and rats) diet and the guinea pigs on Wayne guinea pig diet containing 42 mg of vitamin C added per 100 g.

The animals were anesthetized by intramuscular or intraperitoneal injection of sodium nembutal. The solution contained 15 mg per ml, and 0.4 ml was injected for each 100 g of body weight.

In the spreading method, 0.05 ml (or 0.03 ml) of 0.4 % solution of Evans blue prepared in 0.154 M sodium chloride at pH 7.3 was injected intradermally in the abdominal region over a period of about 30 sec using a hypodermic syringe with a 28 or 29 gauge needle. Three control and three experimental injections were made on each animal. Substances under test were added to the control solution. The area of the spots was measured by tracing the contours on semi-transparent paper and weighing the cut-out surfaces corresponding to the spots [2].

In the infusion method, a 27 gauge hypodermic needle was attached with rubber tubing to a constant pressure burette (MARIOTTE's flask). The pressure was adjusted to give a free flow of about 3 ml per min. The needle was inserted under the skin of the upper dorsal calf region of the animal. Saline (0.154 M) or sodium phosphate solution (0.110 M) adjusted to

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[2] The area of spots determined by this procedure has been found to be in agreement with those measured by a planimeter.

pH 7.3 was allowed to flow from the burette into the animal for 15 minutes; after 5 minutes, 0.5 ml of test solution (in the same solvent) was injected from a syringe into the blebs formed on each calf. This injection was performed over a period of 30 seconds using a 25 gauge needle. The volumes of flow during the first and third 5 minute periods were measured. The ratio of the volume for the third 5 minutes to the volume for the first 5 minutes was used as a basis for comparison. The percentage of change was calculated with respect to the control ratio obtained with injections of the saline or phosphate alone. Both legs of each animal were used to test each examined substance or control solution; thus two values were generally obtained on each animal.

The flavonoids were obtained through the courtesy of the Sunkist Growers, Research Dept., Corona, California. They were hesperidin methyl chalcone and orange « bioflavonoid complex »; the latter was an extract of the skin of this fruit. The rutin, flavone and catechin were supplied by the Nutritional Biochemical Corp., Cleveland, Ohio. The dehydroascorbic acids were prepared by oxidation with purified Norite as described earlier (FABIANEK, 1961 *b*). All solutions were prepared just prior to use and adjusted to pH 7.3. The saturated rutin solution was prepared by dissolving 5 mg in 1 ml of boiling water.

Results

For the spreading test, the effect of the volume of the Evans blue solution on the size of the spot was studied. As shown in Fig. 1, the area at 30 min. was proportional to the logarithm of the injected volume. For most work, an injection volume of 0.05 ml of dye solution was found most practical and was used in the present work.

For dye alone in saline, eighteen male rats had an average spot area at 30 min. of 120 ± 13 mm² (S. D.) and 28 female rats 125 ± 15 mm². Generally the females had greater rates of spreading, but this difference was not significant when examined with the Student *t* test ($P > 0.05$).

The effect of ascorbic acid on the rate of spreading of the Evans blue dye in the dermis of rats is shown in Table 1. Concentrations from 0.1 mg per ml to 40 mg per ml in isotonic

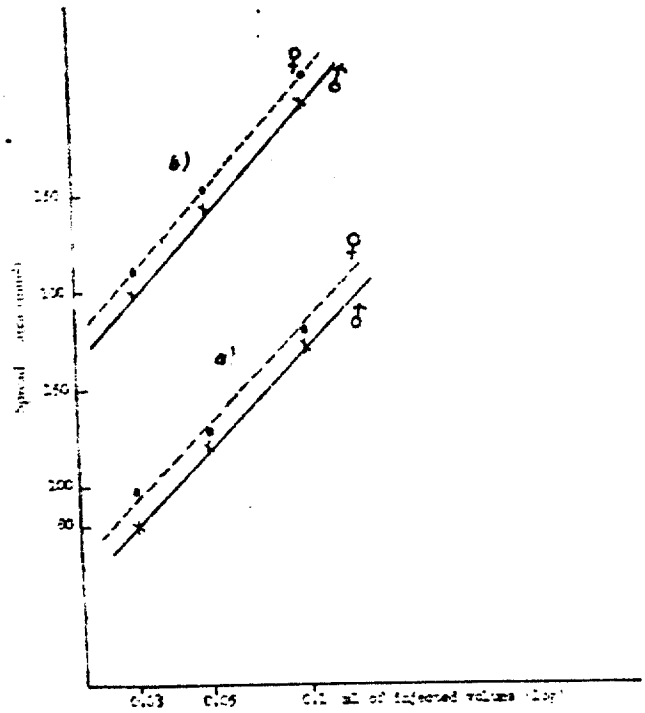

FIG. 1. — Effect of injected volume on spreading area in male and female rats.
a) Evans blue;
b) Evans blue and L-ascorbic acid 1 mg/ml. in isotonic saline at 30 min.
Each point is an average value obtained from 5 animals.

saline were studied. The size of the spots was measured at 30, 60 and 120 minutes with and without ascorbic acid in each animal.

As shown in Table I, at concentrations of ascorbic acid of 1 to 10 mg per ml. a significant increase in the size of the spread area took place, with isotonic saline as the control. The increase was about 10 to 15 %.

A calculation of the significance of a comparison of five animals in an experimental group was made. Each animal had three control and three experimental injections. The average standard deviation was ± 14 mm² at 30 min. The Student *t* test showed that 11 mm² difference from the control area was significant at the 0.01 level [3]. In several groups

[3] In terms of ratios, these correspond to 9 % and 12 %, respectively.

TABLE I

TABLE I

Effect of ascorbic acid concentration on rate of spreading of dye in rat dermis

Ascorbic acid conc. (mg/ml)	Number of animals & sex	Average weight (g)	Spot area (mm^2) ± S. D.						Ratio		
			Control			L-Ascorbic acid					
			30′	60′	120′	30′	60′	120′	30′	60′	120′
.1	5 F	260	127 ± 13	136 ± 12	152 ± 20	133 ± 15	142 ± 13	159 ± 16	1.04	1.04	1.05
.5	6 F	255	122 ± 11	140 ± 11	156 ± 13	126 ± 11	144 ± 17	160 ± 14	1.03	1.03	1.03
1.0	11 F	250	122 ± 15	136 ± 16	158 ± 17	141 ± 21**	155 ± 13**	175 ± 18**	1.15	1.14	1.11
1.0[a]	5 F	270	120 ± 12	134 ± 12	160 ± 12	138 ± 13**	153 ± 15**	187 ± 19**	1.15	1.15	1.17
1.0	6 M	250	119 ± 10	122 ± 14	—	142 ± 10**	150 ± 11**	—	1.19	1.23	—
2.0	5 F	260	132 ± 12	149 ± 17	159 ± 23	141 ± 14	162 ± 20	177 ± 23*	1.07	1.13	1.11
5.0	5 F	265	119 ± 14	128 ± 14	157 ± 18	135 ± 17**	146 ± 15	177 ± 18	1.13	1.14	1.13
10.0	5 F	260	130 ± 17	144 ± 15	169 ± 25	145 ± 20*	162 ± 25*	187 ± 29**	1.14	1.19	1.18
40.0	5 M	270	120 ± 12	—	148 ± 13	130 ± 10*	—	158 ± 12*	1.09	—	1.07

[a] 0.4 % Evans blue in water, at *p*H 7.3.
* $P < 0.05$.
** $P < 0.01$.

examined, the distribution of the spots was normal. These distributions are shown in Figure 2 (*a*, *b*).

When water was used rather than saline as the solvent, the results with the spreading method were the same as with saline. The ratio of spot areas obtained with phosphate buffer (0.110 M) compared with isotonic saline (both at pH 7.3) was 1.08 at 30 min. for female rats ($P < 0.05$).

The effect of ascorbic acid on the infusion rate is shown in Table II. In the first series, the ascorbic acid was dissolved in phosphate buffer, which used alone was the control. The results are given as the ratio of the volume infused during the third

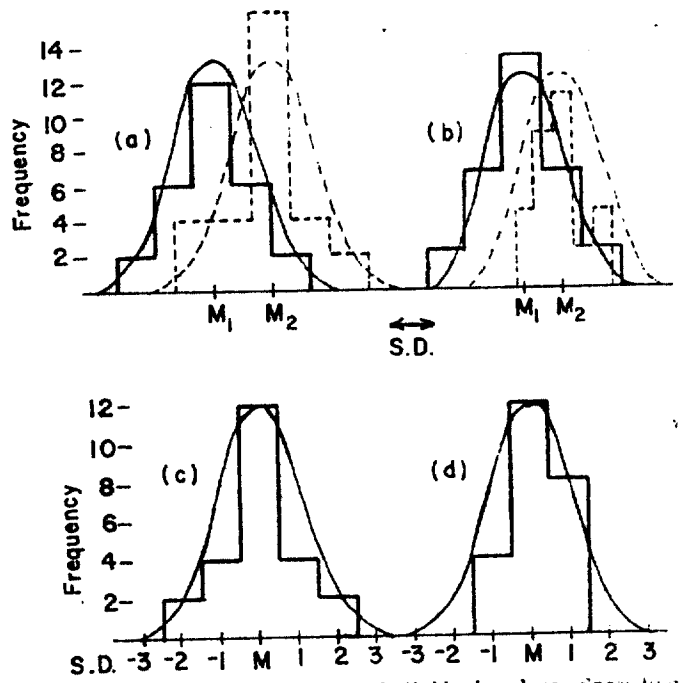

FIG. 2. — Frequency distribution for individual values. Spreading method (areas in mm²) in females (S. D. ± 12) :

a) 1 mg/ml of L-ascorbic acid in 0.4 % Evans blue in water, - - - - - - - - - - -, control, ————————; $P < 0.01$.

b) 40 mg/ml of L-ascorbic acid (males, - - - - - - - - - -; control, ——————; $P < 0.05$.

Infusion method for ratios of 3rd 5 min./1st 5 min. :

c) Saline alone in females (S. D. ± 13).

d) Saline alone in males (S. D. ± 10).

Each interval is one standard deviation from mean (M) except *b*) for L-ascorbic acid with 1/2 S. D.

5 min. to that during the first 5 min. On the basis of a standard deviation averaging ±0.18, the ratios for two groups of six animals (two measurements for each animal) would have to differ by 15 % to be significant at the 0.05 level and 20 % at the 0.01 level (Student *t* test). The distributions about the average were plotted for several experiments, and were approximately normal (Fig. 2, *c*, *d*). Table II also includes data on the effect of ascorbic acid in sodium chloride solution. The ratio of infusion rates was lower in saline alone than in phosphate buffer alone (Table II). For saline solutions female rats had infusion ratios significantly less than males ($P < 0.05$). L-Ascorbic acid at 1 to 10 mg/ml generally enhanced the rate of infusion. However, 40 mg/ml of ascorbic acid had no effect.

TABLE II

Effect of concentration of L-*ascorbic acid tested by infusion method in rats (0.110 M phosphate buffer or 0.154 M sodium chloride as solvent)*

Ascorbic acid concentration (mg/ml)	No. & sex of animals		Ratio : 3rd five min. / 1st five min. ± S. D.	% Increase (+) or decrease (—)
	M	F		
Phosphate alone	8	—	1.12 ± .23	—
Phosphate alone	—	9	1.07 ± .10	—
0.05	3	—	1.18 ± .11	+5
0.1	2	2	1.19 ± .10	+9
0.5	2	3	1.22* ± .18	+11
1.0	—	8	1.17 ± .20	+9
5.0	3	5	1.18 ± .25	+8
10.0	4	3	1.40** ± .29	+28
Saline alone	—	12	0.88 ± .13	—
Saline alone	15	—	1.00 ± .10	—
1	16	—	1.12 ± .25	+12
2	4	—	1.18* ± .23	+18
10	6	—	1.19 ± .42	+19
40	8	—	0.95 ± .23	—5

** $P < 0.01$.
* $P < 0.05$.

TABLE III

Effects of different forms of ascorbic acid in rats by spreading test and by infusion method

Substance (1 mg/ml in saline)	Spreading method (after 30 min.)			Infusion method			
	Number animals	Spot area ratio [a] substance/dye	% Increase (+) or decrease (—)	Number & sex of animals M	Number & sex of animals F	Flow ratio : [a] 3rd five min. / 1st five min.	% Increase (+) or decrease (—)
NaCl (0.154 M)	—	(1.00)	—	15		1.00 ± 0.10	—
					12	0.88 ± 0.13	—
L-Ascorbic acid	17	1.16**	+16	16		1.12 ± 0.25	+12
L-Dehydroascorbic acid	5	0.79**	—21[b]	6	6	0.93 ± 0.13	— 1
D-Isoascorbic acid	6	1.06	+ 9	5		1.22** ± 0.14	+22
		1.18**	+18[c]				
D-Dehydroisoascorbic acid	6	0.92*	— 8	5		0.84** ± 0.03	—16

[a] Mean ± standard deviation.
[b] For a volume of 0.03 ml in 0.110 M phosphate buffer
[c] After 150 min.
** $P < 0.01$.
* $P < 0.05$.

L-Ascorbic acid was compared with D-isoascorbic acid and the oxidized forms of both by the spreading test and by the infusion method. The comparisons were made for concentrations of 1 mg per ml in isotonic sodium chloride. The results are given in Table III.

The D-isoascorbic acid increased the spot size and rate of infusion, but seemed to act somewhat more slowly in the spreading test, so that the difference was significant only at 120 min. The oxidized forms seemed generally to decrease the spreading ratios and rate of infusion, although for the L-dehydroascorbic acid the rate of infusion was the same as for the control.

The effects of several flavonoids on the spreading test are given in Table IV. Catechin and rutin were tested by themselves and in the presence of ascorbic acid. The solvent was isotonic sodium chloride. Most of the data are for rats, but a few experiments with guinea pigs are given. The flavonoids by themselves had only a negligible effect as compared with the controls. As a group, they may have had a slight inhibitory action. In the presence of abscorbic acid, however, rutin and catechin had a strong inhibitive action with the rutin showing the strongest action for both rats and guinea pigs. Flavone and hesperidin methyl chalcone had no significant effect (Table IV).

The same systems were tested for their effect on the rate of infusion. The results are given in Table V. Rutin and catechin alone had only a slight inhibitive effect with respect to the saline control. In the presence of ascorbic acid, rutin showed a strong inhibition and more than reversed the effect of ascorbic acid. This was observed for both rats and guinea pigs. Catechin had a weaker inhibitive action.

Discussion

The effects of ascorbic acid and flavonoids on tissue « permeability » were measured by two methods. One involved the rate of spreading of Evans blue dye injected intradermally into rats and guinea pigs. The other measured the rate of infusion of isotonic saline or phosphate buffer injected subdermally in rats and a few guinea pigs.

Ascorbic acid increased the rate of spreading and of infusion when used at a concentration of 1 to 10 mg. per ml. At a high

TABLE IV

Effect of flavonoids on spreading test using rats and guinea pigs

Substance	Number & sex of animals		Area (mm²) of spots with				% Increase (+) Decrease (—)	
			Dye alone at		Substance + dye at			
	M	F	30′	60′	30′	60′	30′	60′
L-Ascorbic acid (1 mg/ml)	6		119	122	142**	150**	+19	+23
Flavone (5 mg/ml)[a]	3	11	122	136	141**	155**	+15	+14
Rutin (1 mg/ml)	6	3	115	127	117	124	+2	—2
Rutin (half sat.)	3		—	143	—	143	—	0
Flavone (5 mg/ml)[b] + L-Ascorbic acid (1 mg/ml)	6		127	142	116	134	—9	—6
Rutin (sat. sol.) + L-Ascorbic acid (1 mg/ml)	6		122[c]	133[c]	129	145	+6	+9
Catechin (5 mg/ml)	5	5[e]	124[c]	154[cd]	93**	120[d]**	—25	—23[d]
Catechin (5 mg/ml) + L-Ascorbic acid (1 mg/ml)	6		238[c]	—	171**	—	—26	—
Hesperidin methyl chalcone (1 mg/ml)	1[e]	3[e]	95	—	93	—	—2	—
Orange skin extract (1 mg/ml) + L-Ascorbic acid (1 mg/ml)	3	3	128[c]	138[c]	111**	115**	—13	—14
	6		203[c]	—	187	—	—8	—
			111	134	111	134	0	0
			122[c]	—	113	—	—7	—

[a] Dissolved in saline containing 0.1 ml of « Tween 80 » per 10 ml.
[b] Dissolved in a mixture of saline + « Tween 80 » (5 : 1).
[c] Dye + L-Ascorbic acid was the control.
[d] At 120 min.
[e] Guinea pigs; other data for rats.
** [illegible]

TABLE V

Effect of flavonoids on infusion test using rats and guinea pigs

Substance	No. & sex of animals		Ratio : 3rd five min. / 1st five min.	%. Change with regard to :	
	M	F		Saline control	L-Ascorbic acid
Control (saline alone 0.154 M)	12		1.00 ± .10	—	—
		12	0.88 ± .13	—	—
	9[a]		0.95 ± .17	—	—
		7[a]	0.90 ± .12	—	—
L-Ascorbic acid (1 mg/ml)	16		1.12 ± .26	+12	—
	8[a]	7[a]	1.11** ± .12	+20	—
Rutin (sat. sol.)	6		0.90 ± .16	—10	—
Rutin (sat. sol.) + L-Ascorbic acid (1 mg/ml)	7		0.77** ± .20	—23	—33
	5[a]		0.83** ± .20	—11	—28
L-Dehydroascorbic acid (1 mg/ml)	6	6	0.93 ± .12	—1	—
Rutin (sat. sol.) + L-Dehydroascorbic acid (1 mg/ml)	6		0.88 ± .12	—12	—
Catechin (5 mg/ml)	5		0.93 ± .10	—7	—
Catechin (5 mg/ml) + L-Ascorbic acid (1 mg/ml)	9		0.99 ± .25	—1	—14

[a] Guinea pigs; other data are for rats.
** $P < 0.01$

concentration, 40 mg per ml, the effect was reduced or not apparent. This effect of ascorbic acid on enhancing «permeability» about 20 % was shown first by McCLEAN and HALE (1941) for rabbits using a spreading test with hemoglobin as the indicator and ascorbic acid at a concentration of 17.6 mg per ml. PARROT and FASQUELLE (1949) found a similar effect for ascorbic acid (2.5 mg per ml) in rabbits using a spreading test involving India ink as the indicator.

However, DAUBENMERKL (1951), also using rabbits and a method based on the rate of infusion of saline into cutaneous and subcutaneous tissues, found ascorbic acid active only if mixed with hydrogen peroxide in equimolecular proportions, where asascorbic acid without H_2O_2 or H_2O_2 without ascorbic acid had variable effects never as typical or as pronounced as with a mixture of these substances (DAUBENMERKL, 1951).

Unfortunately the author did not give numerical data concerning the effect of ascorbic acid alone. The discrepancy between these results and those reported in the earlier and present work might also be explained by the high concentration of ascorbic acid (44 mg per ml) used by DAUBENMERKL.

Both methods, in the present work, may have shown an effect of sex. Female rats had larger spots for the same amount of injected dye, on the average (Fig. 1). The average area was nearly always greater for females, but usually the variation in any one experiment was too great to be significant by application of the Student *t* test to any single set of averages. However, the effect of ascorbic acid was the same for males and females as shown by their ratios. The same effects for guinea pigs were shown earlier (FABIANEK, HERP and PIGMAN, 1963). The rate of infusion for females was less than that for males, which for males was the same for the third 5 minute period as for the first 5 minute period.

D-Isoascorbic acid behaved much like L-ascorbic acid in both methods. The oxidized forms behaved differently in the two methods and either had no effect or were inhibitory. The oxidized forms, however, are very unstable and may have been destroyed, although used as soon as possible after preparation.

Several flavonoids were tested by both methods and generally had little or no activity except for rutin and catechin which were inhibitors of the action of ascorbic acid. PARROT and

FASQUELLE (1949) found that catechin inhibited the spread of India ink in rabbits and that the inhibition was greater in the presence of ascorbic acid.

Flavonoids have been studied earlier as factors inhibiting the effect of hyaluronidase. Their direct effect on dermal connective tissue was observed, only incidentally, in control animals. Nevertheless, the reported results have been lacking in coherence. In LEVITAN's (1948) experiments, rutin (200 mg injected intraperitoneally in rats) inhibited markedly the spreading activity of intradermally injected hyaluronidase and produced also a decrease in the spread of the control ink and saline. Contrariwise in similar experiments of ELSTER (1949), rutin (200 mg orally or intraperitoneally) failed to produce an effect. In experiments of RODNEY *et al.* (1950), catechin tested *in vivo* appeared markedly more active than rutin as an inhibitor of hyaluronidase.

RODNEY *et al.* (1950), concluded from a study of a series of flavonoids that the free hydroxyl groups in both the 3′ and 4′ positions were necessary to confer inhibitory action against hyaluronidase. The present work suggests that two adjoining hydroxyl or one hydroxyl and one keto group situated on the flavonoid nucleus appear necessary. Such compounds have also been shown by CLARK and GEISSMAN (1949) to have chelating activity. However, in the *in vivo* experiments of the present work rutin was more active than catechin. Since the OH in position 3 is blocked by a glycosidic linkage in rutin, these results suggest that this linkage possibly may be hydrolyzed during the *in vivo* tests.

In the present work, hesperidin methyl chalcone and flavone which lack free adjoining hydroxyl groups were devoid of any activity. The methyl group in hesperidin and hesperidin chalcone presumably did not split off *in vivo*. The inactivity of the orange skin extracts is not surprising. Hesperidin is the main flavonoid in lemon and in orange extract (BRUCKNER and SZENT-GYÖRGYI, 1936). Eriodictyol glycoside (containing two adjoining hydroxyl groups) is another flavonoid found in « citrin », but in lower concentration than hesperidin (BRUCKNER and SZENT-GYÖRGYI, 1936).

The activity of the compounds tested in these *in vivo* « permeability » tests is closely paralleled by their effects on the *in vitro* degradation of hyaluronic acid. In the *in vitro* expe-

riments, ascorbic acid caused a marked decrease in the viscosity of hyaluronic acid (PIGMAN *et al.*, 1959; 1961). The action was quite pronounced at a concentration of 1 mg per ml, and high concentrations (40 mg per ml) were less effective (unpublished results of Drs. G. MATSUMURA and A. HERP). Rutin and catechin were inhibitors of the *in vitro* reaction. The action of ascorbic acid in phosphate buffer was greater than for sodium chloride solutions for both the *in vitro* and *in vivo* systems.

Summary

1. Reduced and oxidized forms of L-ascorbic acid and D-iso-ascorbic acid and several flavonoids were tested for their activity on the spreading of Evans blue dye intradermally injected and on the rate of subdermal infusion of isotonic saline or phosphate buffer under constant pressure. White rats and guinea pigs were used.

2. The two methods generally showed similar effects. In the spreading test the area of spot was directly proportional to the logarithm of the injected volume. Reduced forms of ascorbic acids increased both the spreading and rate of infusion. High concentrations of L-ascorbic acid showed less activity than optimal concentrations. The oxidized forms of ascorbic acid had no effect or were inhibitors. Rutin and catechin had no effect or acted as slight inhibitors. With L-ascorbic acid, they showed a marked inhibition.

3. The behavior of these substances in the *in vivo* systems generally paralleled their effects on the degradation of hyaluronic acid *in vitro*.

Résumé

1. On a examiné l'effet des formes réduites et oxydées des acides L-ascorbique et D-isoascorbique ainsi que l'effet de quelques flavonoïdes sur la vitesse de diffusion de Bleu d'Evans injecté par voie intradermale et sur la vitesse d'infusion sous-cutanée sous pression constante de l'eau physiologique ou d'un tampon de phosphate isotonique. Les expériences ont été faites sur des rats et cobayes.

2. Les deux méthodes ont en général conduit à des résultats semblables. Dans la méthode par diffusion, la surface des taches

obtenues a été trouvée directement proportionnelle au logarithme du volume injecté. Les formes réduites des acides ascorbiques ont augmenté aussi bien la vitesse de diffusion que celle d'infusion. L'acide L-ascorbique en concentrations élevées était moins actif qu'en concentrations optimales. Les formes oxydées des acides ascorbiques étaient sans effet ou ont produit un léger effet inhibiteur. Il en était de même pour la rutine et la catéchine. Ces dernières substances, associées à l'acide ascorbique, ont produit un effet fortement inhibiteur.

3. Le comportement de ces substances dans le système *in vivo* concordait généralement avec leur effet sur la dégradation de l'acide hyaluronique *in vitro*.

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GROUP 2: ANTIOXIDANTS

Name	Function, Usage	Levels of Use
Ascorbate, Calcium Ascorbate, Sodium	Antioxidants	
	Concentrated milk products	
	Cooked, cured, or comminuted meat food products, per 100 lbs of the fresh, uncured, or comminuted meat and/or by-product	7/8 oz
	Used in the pickle in which pork and beef products are cured or packed per 100 gal, up to	75 oz
Ascorbic Acid (Vitamin C)	Antioxidant	
	Frozen fruit, particularly sliced peaches	0.03 - 0.05%
	Frozen fish dip	0.25 - 2.0%
	Dry milk (wet basis)	0.0025 - 0.01%
	Beer and ale	0.0015 - 0.0075%
	Flavoring oils	0.1 - 0.3%
	Apple juice, soft drinks	0.01 - 0.05%
	Beef briskets	
	Fluid milk	
	Candy	1.0%
	Artificially sweetened jellies and preserves. Up to	0.1%
	Canned mushrooms per ounce of drained weight	37.5 mg
	Cooked, cured, comminuted meat food products, per 100 lbs of product, up to	3/4 oz
	Pickle in which pork or beef products are cured or packed, per 100 gal	75 oz
Ascorbyl Palmitate	Candy	0.06%
Butylated Hydroxyanisole (Butylated Hydroxyanisole; mixture of 2 and 3 tertiary-butyl-4-hydroxyanisole)	Antioxidant	
	Beverages, ice cream, ices	0.00003 - 0.0002%
	Candy, as percent of fat, up to	0.1%
	Baked goods	0.0002 - 0.001%
	Gelatin dessert	0.00004 - 0.00015%
	Potatoes	0.0002 - 0.0005%
	Glacéed fruits, mixed, diced. Up to	0.0032%
	Potato flakes, sweet-potato flakes. Alone or with butylated hydroxytoluene and/or propyl gallate, up to	0.005%
	Chewing gum base. Alone or with butylated hydroxytoluene and/or propyl gallate, up to	0.1%
	Defoamer component in yeast and beet-sugar production. Up to	0.1%
	Dry breakfast cereals. Total antioxidant, alone or with butylated hydroxytoluene and/or propyl gallate, up to	0.005%
	Potato granules. Total antioxidant, up to	0.001%
	Dry yeast. Up to	0.1%
	Dry mixes for beverages and desserts. Up to	0.001%
	Beverages and desserts prepared from dry mixes. Up to	0.0002%
	Lard and shortening. Up to	0.01%
	Unsmoked dry sausage. Up to	0.003%
	Emulsion stabilizers for shortenings, alone or with butylated hydroxytoluene	0.02%
	Unsmoked dry sausage	0.003%

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Name	Function, Usage	Levels of Use
Butylated Hydroxytoluene (Di-tertiary-Butyl-para-Cresol) (2,6-di-tertiary-Butyl-4-methyl phenol)	Antioxidant	
	Chewing-gum base. Alone or with butylated hydroxyanisole and/or propyl gallate, up to	0.1%
	Potato flakes and sweet-potato flakes. Up to	0.005%
	Defoamer component in yeast and beet-sugar production. Up to	0.1%
	Potato granules. Total antioxidant, up to	0.001%
	Dry breakfast cereals. Total antioxidant, up to	0.005%
	Emulsion stabilizers for shortenings. Alone or with butylated hydroxyanisole, up to	0.02%
	Enriched rice. Up to	0.0033%
	Animal fats and shortenings containing animal fats. Up to	0.01%
Carbon Monoxide (Combustion product gas)	Removing and displacing oxygen	
	Carbon monoxide, up to 4.5% of gas	
Dilauryl Thiodipropionate	Antioxidant	
	General food use, per cent of fat or oil content, up to	0.02%
Erythorbate, Sodium (Sodium Isoascorbate) Erythorbic Acid (Isoascorbic Acid)	Antioxidants	
	Pickling brine. Per 100 gal, up to	7.5 oz
	Meat products. Per 100 lbs, up to	3/4 oz
	Brine, solution. Up to	10.0%
	Beverages	0.006 - 0.01%
	Baked goods	0.02 - 0.05%
	Cured cuts; cured comminuted product to accelerate color fixing in curing, per 100 lbs, Sodium erythorbate, up to	7/8 oz
	Erythorbic acid, up to	3/4 oz
	Pickle in which pork and beef products are cured or packed, per 100 gal	75 oz
Gas, Combustion product (from the controlled combustion in air of butane, propane, or natural gas)	Removing and displacing oxygen in the processing, storage, or packaging of citrus products, vegetable fats, vegetable oils, coffee, and wine	
Gum Guaiac	Antioxidant in edible fats or oils (Equivalent antioxidant activity - 0.01%)	
	Beverages	0.04 - 0.06%
	Rendered animal fat or a combination of such fat and vegetable fat. Up to	0.1%
Lecithin	Antioxidant	
	Prepared breakfast cereal	0.0025 - 0.005%
	Candy	0.06 - 0.25%
	Sweet chocolate, milk chocolate, etc.	
	Bread, rolls, buns, etc.	
	Oleomargarine. Up to	0.5%.
Nordihydroguaiaretic Acid (Dihydroguaiaretic acid, normal)	Antioxidant	
	Prepared pie-crust mix	0.01%
	Candy (of fat)	0.01%
	Lard, butter, ice cream	
	Gassed cream (pressure-dispensed whipped cream)	
	General food use. Per cent of fat or oil content including essential (volatile) oil content, up to	0.02%
	Rendered animal fat or a combination of such fat and vegetable fat. Up to	0.01%

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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, %
0124	MANGANESE SULFATE	83	FORMULAS(B)	4	.00237	.00554
0126	MANNITOL	83	FORMULAS(B)	*	.00003	.00031
0130	MONO- AND DIGLYCERIDES	83	FORMULAS(B)	*	.04091	.11218
0136	NIACIN	81	BAKED GOODS(B)	*	.01300	.01500
0137	NIACINAMIDE	82	CEREALS(B)	*	.00510	.00541
		83	FORMULAS(B)	6	.00065	.00093
		93	COMB DINNER(B)	*	.00900	.00900
0148	POTASSIUM BICARBONATE	83	FORMULAS(B)	*	.01682	.01682
0151	POTASSIUM CHLORIDE	83	FORMULAS(B)	4	.06195	.06195
0152	POTASSIUM CITRATE	83	FORMULAS(B)	5	.15969	.25934
0154	POTASSIUM HYDROXIDE	83	FORMULAS(B)	*	.05414	.05414
0155	POTASSIUM IODIDE	83	FORMULAS(B)	6	.00013	.00015
0167	PYRIDOXINE HYDROCHLORIDE	81	BAKED GOODS(B)	*	.00500	.00580
		82	CEREALS(B)	*	.00018	.00055
		83	FORMULAS(B)	7	.00007	.00479
0169	RIBOFLAVIN	81	BAKED GOODS(B)	*	.00290	.00340
		82	CEREALS(B)	*	.00005	.00143
		83	FORMULAS(B)	7	.00011	.01124
		93	COMB DINNER(B)	*	.00070	.00070
0180	SODIUM ASCORBATE	83	FORMULAS(B)	4	.01620	.02004
0182	SODIUM BICARBONATE	81	BAKED GOODS(B)	*	.65000	.79000

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, %
AMMONIUM SULFATE (CONT.)	08	PROCSD FRUT(R)	*	.00000	.00000
NAS 0019	19	SWEET SAUCE(R)	*	.00000	.00000
	20	GELATIN PUD(R)	*	.07500	.10000
ASCORBIC ACID	01	BAKED GOODS(R)	11	.02751	.02986
NAS 0022 FEMA 2109	02	BREAK CERLS(R)	4	.01277	.05097
	05	MILK PRODS(R)	9	.00892	.01370
	07	FROZN DAIRY(R)	5	.01658	.01658
	08	PROCSD FRUT(R)	32	.03156	.06080
	10	MEAT PRODS(R)	14	.04592	.04839
	11	POULTRY(R)	*	.04600	.04600
	13	FISH PRODS(R)	*	.01800	.03000
	14	PROCSD VEGS(R)	8	.01588	.02392
	15	CONDM RELSH(R)	*	.00569	.01000
	18	JAM JELLY(R)	*	.12660	.12660
	19	SWEET SAUCE(R)	*	.19637	.22844
	20	GELATIN PUD(R)	8	.02368	.03019
	21	SOUPS(R)	*	.01825	.05171
	23	BEV TYPE I(R)	35	.00689	.05082
	24	BEV TYPE II(R)	*	.01200	.01600
	27	GRAVIES(R)	*	.19406	.32517
	30	HARD CANDY(R)	*	.24784	.88703
	34	INS COF TEA(R)	*	.00847	.00847
	49	MISC UNCLAS(R)	*	.00997	.03000
ASCORBYL PALMITATE	04	FATS OILS(R)	*	.01000	.03000
NAS 0023	34	INS COF TEA(R)	*	********	.00001
BEESWAX	01	BAKED GOODS(R)	*	********	********
NAS 0026	08	PROCSD FRUT(R)	*	.00100	.00100
	16	SOFT CANDY(R)	4	.04398	.05385
	30	HARD CANDY(R)	*	.01154	.01355
	31	CHEWING GUM(R)	*	********	********
BEESWAX, BLEACHED	01	BAKED GOODS(R)	4	.00034	.00064
NAS 0027 FEMA 2126	07	FROZN DAIRY(R)	4	.00011	.00030
	16	SOFT CANDY(R)	7	.04855	.04903
	17	CONF FROST(R)	*	.00318	.00482
	19	SWEET SAUCE(R)	*	.00005	.00050
	20	GELATIN PUD(R)	*	.00006	.00020
	23	BEV TYPE I(R)	6	.00006	.00019
	24	BEV TYPE II(R)	*	.00020	.00080
	31	CHEWING GUM(R)	*	.03390	.03390

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, %
CITRIC ACID (CONT.) NAS 0068 FEMA 2306	49	MISC UNCLAS(R)	*	.13368	.26744
COPPER GLUCONATE NAS 0069	05	MILK PRODS(R)	*	.00150	.00500
	08	PROCSD FRUT(R)	*	.07440	.07500
	16	SOFT CANDY(R)	*	.00450	.00500
	22	SNACK FOODS(R)	*	.00000	.00000
	31	CHEWING GUM(R)	*	.00471	.00471
L-CYSTEINE NAS 0071 FEMA 3285	01	BAKED GOODS(R)	8	.00703	.01726
	03	OTHER GRAIN(R)	*	.00400	.01800
	07	FROZN DAIRY(R)	*	.00750	.01500
	08	PROCSD FRUT(R)	*	.00053	.00150
	10	MEAT PRODS(R)	10	.00937	.01859
	11	POULTRY(R)	*	.00500	.02000
	14	PROCSD VEGS(R)	*	.00264	.00970
	15	CONDM RELSH(R)	*	.00007	.00008
	16	SOFT CANDY(R)	*	.00800	.01600
	20	GELATIN PUD(R)	*	.00800	.01600
	21	SOUPS(R)	*	.01128	.02797
	23	BEV TYPE I(R)	*	.00081	.00204
	24	BEV TYPE II(R)	*	.00002	.00002
	26	RECONST VEG(R)	*	.01500	.03500
	27	GRAVIES(R)	*	.00995	.02486
	48	SEAS FLAVRS(R)	*	.00030	.00400
DL-CYSTINE FEMA 3286	01	BAKED GOODS(R)	*	.02000	.03000
	07	FROZN DAIRY(R)	*	.00750	.01500
	10	MEAT PRODS(R)	*	.01240	.01916
	15	CONDM RELSH(R)	*	.00300	.01000
	16	SOFT CANDY(R)	*	.00800	.01600
	20	GELATIN PUD(R)	*	.00800	.01600
	23	BEV TYPE I(R)	*	.00500	.01000
DIACETYL TAR MONODIGLY NAS 0076	01	BAKED GOODS(R)	*	.17720	.17723
	04	FATS OILS(R)	*	.05350	.05350
	17	CONF FROST(R)	*	.00167	.00222
	28	IMIT DAIRY(R)	*	.08400	.08400
ERYTHORBIC ACID NAS 0078 FEMA 2410	01	BAKED GOODS(R)	*	.00026	.00077
	04	FATS OILS(R)	*	.00004	.06000
	09	FRUIT ICES(R)	*	.00400	.00400
	10	MEAT PRODS(R)	*	.05366	.05377

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, %
ERYTHORBIC ACID (CONT.)	14	PROCSD VEGS(R)	*	.00001	.00002
NAS 0078 FEMA 2410	16	SOFT CANDY(R)	*	.00020	.00060
	23	BEV TYPE I(R)	5	.00510	.00975
	27	GRAVIES(R)	*	.01500	.03000
ETHYL FORMATE	01	BAKED GOODS(R)	20	.02752	.02966
NAS 0079 FEMA 2434	07	FROZN DAIRY(R)	18	.00923	.01020
	10	MEAT PRODS(R)	*	.00025	.00100
	16	SOFT CANDY(R)	19	.01745	.01838
	20	GELATIN PUD(R)	14	.00923	.01495
	23	BEV TYPE I(R)	33	.00478	.00550
	24	BEV TYPE II(R)	7	.00019	.00038
	30	HARD CANDY(R)	10	.01158	.01158
	31	CHEWING GUM(R)	5	.00915	.01479
	49	MISC UNCLAS(R)	*	.00887	.05738
FERRIC PHOSPHATE	01	BAKED GOODS(R)	*	.00693	.01028
NAS 0080	02	BREAK CERLS(R)	6	.06385	.12875
	03	OTHER GRAIN(R)	5	.00763	.01258
	05	MILK PRODS(R)	5	.00614	.00886
	15	CONDM RELSH(R)	*	.00100	.00100
	23	BEV TYPE I(R)	*	.01602	.01602
	27	GRAVIES(R)	*	.01090	.01090
	28	IMIT DAIRY(R)	*	.01909	.01909
FERRIC PYROPHOSPHATE	02	BREAK CERLS(R)	*	.05000	.20000
NAS 0081	05	MILK PRODS(R)	*	.00273	.00273
	19	SWEET SAUCE(R)	*	.06000	.06000
	22	SNACK FOODS(R)	*	.00000	.00000
	23	BEV TYPE I(R)	*	.00000	.00000
	26	RECONST VEG(R)	*	.00620	.00620
FERRIC SODIUM PYROPHOS	01	BAKED GOODS(R)	*	.02098	.02098
NAS 0082	02	BREAK CERLS(R)	5	.14871	.50670
	03	OTHER GRAIN(R)	*	.01945	.02455
	05	MILK PRODS(R)	*	.02856	.02931
	10	MEAT PRODS(R)	*	.00160	.00160
	11	POULTRY(R)	*	.00900	.00900
	13	FISH PRODS(R)	*	.00900	.00900
	19	SWEET SAUCE(R)	*	.16300	.16300
FERROUS GLUCONATE	15	CONDM RELSH(R)	*	.00200	.00200
NAS 0083					

TABLE 12, 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 A	HIGH B
ASCORBIC ACID NAS 0022	24	BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	**********	.012000	**********
				12-23 MO.	**********	.024000	**********
				2-65+ YR.	3.900000	11.328000	5.200000
ASCORBIC ACID NAS 0022	27	GRAVIES(R)	*	0-5 MO.	.194060	.582180	.325170
				6-11 MO.	2.718840	7.568340	4.552380
				12-23 MO.	6.586160	19.794120	11.706120
				2-65+ YR.	16.106580	41.334780	26.969110
ASCORBIC ACID NAS 0022	30	HARD CANDY(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	.247840	.743520	.867030
				12-23 MO.	.743520	2.230560	2.661090
				2-65+ YR.	1.487040	4.213280	5.322180
ASCORBIC ACID NAS 0022	34	IRS COF TEA(R)	*	0-5 MO.	.016940	.270510	.016940
				6-11 MO.	.448910	1.109570	.448910
				12-23 MO.	.525140	1.857280	.525140
				2-65+ YR.	10.257170	21.971180	10.257170
ASCORBIC ACID NAS 0022	83	FORMULAS(R)	4	0-5 MO.	40.049010	73.369500	47.568690
				6-11 MO.	8.160120	38.879870	9.692280
				12-23 MO.	2.624800	.739660	3.117400
ASCORBIC ACID NAS 0022	34	PROCSD FRUT(B)	*	0-5 MO.	31.429200	74.858640	60.225000
				6-11 MO.	32.286360	101.666700	61.867500
				12-23 MO.	8.000160	33.476860	15.330000
ASCORBIC ACID NAS 0022	90	PUDDINGS(B)	*	0-5 MO.	3.695620	13.519420	4.085880
				6-11 MO.	4.537660	16.794020	5.016840
				12-23 MO.	1.263060	1.450180	1.396440
ASCORBIC ACID NAS 0022	93	COMB DINNER(B)	*	0-5 MO.	9.200000	31.360000	9.200000
				6-11 MO.	20.320000	56.920000	20.320000
				12-23 MO.	6.200000	23.600000	6.200000
ASCORBIC ACID NAS 0022	ALL CATEGORIES	**********	101	0-5 MO.	89.959600	205.856190	130.239730
	**********	**********		6-11 MO.	136.425250	420.772830	219.266570
	**********	**********		12-23 MO.	129.720040	308.845270	229.697640
	**********	**********		2-65+ YR.	212.677200	448.015790	347.130270
ASCORBYL PALMITATE NAS 0023	04	FATS OILS(R)	*	0-5 MO.	.050000	.050000	.130000
				6-11 MO.	.280000	.750000	.840000
				12-23 MO.	.630000	1.200000	1.890000
				2-65+ YR.	1.750000	3.160000	5.250000

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 A	HIGH B
ASCORBYL PALMITATE NAS 0023	34 INS COF TEA(R)	*	0-5 MO.	**********	**********	.000020
			6-11 MO.	**********	**********	.000530
			12-23 MO.	**********	**********	.000620
			2-65+ YR.	**********	**********	.012110
ASCORBYL PALMITATE NAS 0023	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.050000	.050000	.150020
			6-11 MO.	.280000	.750000	.840530
			12-23 MO.	.630000	1.200000	1.890620
			2-65+ YR.	1.750000	3.160000	5.262110
BEESWAX NAS 0026	01 BAKED GOODS(R)	*	0-5 MO	**********	**********	**********
			6-11 MO.	**********	**********	**********
			12-23 MO.	**********	**********	**********
			2-65+ YR.	**********	**********	**********
BEESWAX NAS 0026	08 PROCSD FRUT(R)	*	0-5 MO.	.047000	.120000	.047000
			6-11 MO.	.518000	1.290000	.518000
			12-23 MO.	1.006000	1.997000	1.006000
			2-65+ YR.	1.183000	2.506000	1.183000
BEESWAX NAS 0026	16 SOFT CANDY(R)	4	0-5 MO.	.087960	.879600	.107700
			6-11 MO.	.987560	2.990640	1.184700
			12-23 MO.	1.539300	4.080140	1.884750
			2-65+ YR.	2.550840	7.740480	3.123300
BEESWAX NAS 0026	30 HARD CANDY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.011540	.034620	.013550
			12-23 MO.	.034620	.103860	.040650
			2-65+ YR.	.069240	.196180	.081300
BEESWAX NAS 0026	31 CHEWING GUM(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	**********	**********
			12-23 MO.	**********	**********	**********
			2-65+ YR.	**********	**********	**********
BEESWAX NAS 0026	ALL CATEGORIES ********** ********** **********	10	0-5 MO.	.134960	1.005600	.154700
			6-11 MO.	1.497100	4.315260	1.716250
			12-23 MO.	2.579920	6.191080	2.931400
			2-65+ YR.	3.803090	10.442660	4.387600
BEESWAX, BLEACHED NAS 0027	01 BAKED GOODS(R)	4	0-5 MO.	.011560	.015300	.021760
			6-11 MO.	.086360	.176120	.162560
			12-23 MO.	.185300	.305320	.348800
			2-65+ YR.	.466480	.692920	.878080

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, %
SODIUM ALUMINOSILICATE (CONT.) NAS 0178	19	SWEET SAUCE(R)	*	.38675	.38675
	20	GELATIN PUD(R)	*	.03000	.07000
	21	SOUPS(R)	4	.41969	.42044
	22	SNACK FOODS(R)	*	.63145	.92768
	23	BEV TYPE I(R)	*	.07918	.07918
	27	GRAVIES(R)	*	.06434	.19911
	28	IMIT DAIRY(R)	5	.87533	.87533
	48	SEAS FLAVRS(R)	*	.53938	.53950
SODIUM ALUMINUM PHOS NAS 0179 FEMA 3657	01	BAKED GOODS(R)	23	1.19135	1.56896
	03	OTHER GRAIN(R)	*	.35828	.41925
	06	CHEESE(R)	*	2.21736	2.40518
	10	MEAT PRODS(R)	*	.13580	.13580
SODIUM ASCORBATE NAS 0180 FEMA 3525	01	BAKED GOODS(R)	*	.09412	.13824
	02	BREAK CERLS(R)	8	.09272	.34125
	05	MILK PRODS(R)	6	.02964	.03291
	08	PROCSD FRUT(R)	*	.11950	.15199
	10	MEAT PRODS(R)	14	.03306	.04941
	11	POULTRY(R)	*	.03600	.03600
	13	FISH PRODS(R)	*	.03600	.03600
	23	BEV TYPE I(R)	*	.00000	.00000
	24	BEV TYPE II(R)	10	.00080	.00160
	28	IMIT DAIRY(R)	*	.11250	.11250
	30	HARD CANDY(R)	*	.12000	.30000
SODIUM BENZOATE NAS 0181 FEMA 3025	01	BAKED GOODS(R)	19	.04861	.10961
	02	BREAK CERLS(R)	*	.00465	.00465
	04	FATS OILS(R)	16	.09707	.09799
	05	MILK PRODS(R)	5	.01845	.03207
	06	CHEESE(R)	5	.00004	.00005
	07	FROZN DAIRY(R)	11	.01159	.01549
	08	PROCSD FRUT(R)	18	.06958	.09056
	09	FRUIT ICES(R)	*	.04999	.05011
	10	MEAT PRODS(R)	*	.00417	.00817
	14	PROCSD VEGS(R)	6	.07728	.10000
	15	CONDM RELSH(R)	13	.08039	.10666
	16	SOFT CANDY(R)	12	.02070	.03109
	17	CONF FROST(R)	*	.09377	.09377
	18	JAM JELLY(R)	5	.07483	.09996
	19	SWEET SAUCE(R)	20	.08676	.29170
	20	GELATIN PUD(R)	10	.07229	.09495
	23	BEV TYPE I(R)	49	.03865	.05961
	24	BEV TYPE II(R)	*	.00384	.00384

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, %
PROPYLENE GLYCOL MSTEARAT(CONT.)	23	BEV TYPE I(R)	*	********	.00050
NAS 0309 FEMA 2942	24	BEV TYPE II(R)	*	********	.00050
	28	IMIT DAIRY(R)	*	.30000	.50000
PROTEIN, ANIMAL, HYDROLYZ	03	OTHER GRAIN(R)	*	.01500	.03000
NAS 0310	10	MEAT PRODS(R)	*	.01500	.03000
	11	POULTRY(R)	*	.09379	.10503
	21	SOUPS(R)	*	.03846	.05149
	27	GRAVIES(R)	*	.01500	.03000
PROTEIN, VEGE, HYDROLYZED	01	BAKED GOODS(R)	4	.49040	4.33338
NAS 0311 FEMA 3517	03	OTHER GRAIN(R)	7	.28910	.46046
	04	FATS OILS(R)	*	.54848	.54575
	05	MILK PRODS(R)	*	.24015	.35998
	06	CHEESE(R)	*	.14231	.26610
	10	MEAT PRODS(R)	26	.37506	.58445
	11	POULTRY(R)	14	.20259	.30560
	12	EGG PRODS(R)	*	.22000	.22000
	13	FISH PRODS(R)	*	.24427	.44860
	14	PROCSD VEGS(R)	7	.09469	.11191
	15	CONDM RELSH(R)	5	18.08195	19.99572
	16	SOFT CANDY(R)	*	.05000	.05000
	21	SOUPS(R)	16	.52150	1.23101
	22	SNACK FOODS(R)	4	.04578	.17999
	26	RECONST VEG(R)	*	.77799	3.73915
	27	GRAVIES(R)	18	.85704	1.52816
	28	IMIT DAIRY(R)	*	.51000	1.00000
	48	SEAS FLAVRS(R)	6	3.34339	5.82163
SAUSAGE CASINGS	10	MEAT PRODS(R)	*	2.00000	2.00000
NAS 0313	11	POULTRY(R)	*	.01000	.02000
SODIUM CHLORITE	03	OTHER GRAIN(R)	*	.02000	.02000
NAS 0316					
SODIUM ERYTHORBATE	02	BREAK CERLS(R)	*	.00200	.00750
NAS 0317	05	MILK PRODS(R)	*	.00009	.00024
	10	MEAT PRODS(R)	17	.03663	.39994
	11	POULTRY(R)	*	.01250	.01250
	23	BEV TYPE I(R)	*	.00036	.00036
	24	BEV TYPE II(R)	20	.00139	.00170

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-CYSTINE NAS 0072	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	1.187400	2.126100	1.972760
			6-11 MO.	10.718300	26.858200	17.761120
			12-23 MO.	19.902800	38.715600	32.764320
			2-65+ YR.	46.641600	81.646900	75.493440
DIACETYL TAR MONODIGLY NAS 0076	01 BAKED GOODS(R)	*	0-5 MO.	6.024900	7.974000	6.025820
			6-11 MO.	45.008600	91.789600	45.016420
			12-23 MO.	96.574000	159.125600	96.590350
			2-65+ YR.	243.118400	361.133600	243.159560
DIACETYL TAR MONODIGLY NAS 0076	04 FATS OILS(R)	*	0-5 MO.	.267500	.267500	.267500
			6-11 MO.	1.498000	4.012500	1.498000
			12-23 MO.	3.370500	6.420000	3.370500
			2-65+ YR.	9.362500	16.905000	9.362500
DIACETYL TAR MONODIGLY NAS 0076	17 CONF FROST(R)	*	0-5 MO.	**********	.001670	**********
			6-11 MO.	.001670	.003340	.002220
			12-23 MO.	.003340	.011690	.004440
			2-65+ YR.	.005010	.013360	.006660
DIACETYL TAR MONODIGLY NAS 0076	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	1.176000	1.932000	1.176000
			12-23 MO.	.672000	2.856000	.672000
			2-65+ YR.	.756000	1.260000	.756000
DIACETYL TAR MONODIGLY NAS 0076	ALL CATEGORIES ********** ********** **********	6	0-5 MO.	6.293300	8.243170	6.293320
			6-11 MO.	47.684470	97.737440	47.692640
			12-23 MO.	100.619840	168.413290	100.637290
			2-65+ YR.	253.241510	379.312960	253.284720
ERYTHORBIC ACID NAS 0078	01 BAKED GOODS(R)	*	0-5 MO.	.008840	.011700	.026180
			6-11 MO.	.068040	.134680	.195580
			12-23 MO.	.141700	.233490	.419650
			2-65+ YR.	.356720	.529880	1.056440
ERYTHORBIC ACID NAS 0078	04 FATS OILS(R)	*	0-5 MO.	.000200	.000200	.300000
			6-11 MO.	.001120	.003000	1.680000
			12-23 MO.	.002520	.004200	3.780000
			2-65+ YR.	.007000	.012640	10.500000
ERYTHORBIC ACID NAS 0078	09 FRUIT ICES(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.012000	.032000	.012000
			12-23 MO.	.024000	.084000	.024000
			2-65+ YR.	.028000	.100000	.028000

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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 A	HIGH B
ERYTHORBIC ACID NAS 0078	10 MEAT PRODS(R)	*	0-5 MO.	.590260	1.556140	.591470
			6-11 MO.	11.107620	29.942280	11.130390
			12-23 MO.	16.205320	27.849540	16.238540
			2-65+ YR.	42.069440	69.811660	42.155680
ERYTHORBIC ACID NAS 0078	14 PROCSD VEGS(R)	*	0-5 MO.	.000140	.000420	.000280
			6-11 MO.	.002400	.005600	.004800
			12-23 MO.	.003900	.006530	.007800
			2-65+ YR.	.008500	.014320	.017000
ERYTHORBIC ACID NAS 0078	16 SOFT CANDY(R)	*	0-5 MO.	.000400	.004000	.001600
			6-11 MO.	.004400	.017600	.017600
			12-23 MO.	.007000	.018600	.028000
			2-65+ YR.	.011600	.039200	.046400
ERYTHORBIC ACID NAS 0078	23 DEV TYPE I(R)	5	0-5 MO.	.122400	.183600	.234000
			6-11 MO.	1.157700	3.962700	2.213250
			12-23 MO.	2.764200	8.287500	5.284800
			2-65+ YR.	5.304000	14.162700	10.140000
ERYTHORBIC ACID NAS 0078	27 GRAVIES(R)	*	0-5 MO.	.015000	.045000	.030000
			6-11 MO.	.210000	.585000	.420000
			12-23 MO.	.540000	1.530000	1.080000
			2-65+ YR.	1.245000	3.195000	2.490000
ERYTHORBIC ACID NAS 0078	ALL CATEGORIES ********** ********** **********	11	0-5 MO.	.737240	1.801060	1.183530
			6-11 MO.	12.561280	34.678860	15.673620
			12-23 MO.	19.688640	38.014450	26.862490
			2-65+ YR.	49.030260	87.861400	66.433520
ETHYL FORMATE NAS 0079	01 BAKED GOODS(R)	20	0-5 MO.	.935580	1.238400	1.008440
			6-11 MO.	6.990080	14.255360	7.533640
			12-23 MO.	14.996400	24.712960	16.164700
			2-65+ YR.	37.757440	56.085760	40.693520
ETHYL FORMATE NAS 0079	07 FRCZN DAIRY(R)	18	0-5 MO.	.092300	.378430	.102000
			6-11 MO.	.876850	2.436720	.959000
			12-23 MO.	1.329120	3.119740	1.468500
			2-65+ YR.	2.362880	5.694910	2.611200
ETHYL FORMATE NAS 0079	10 MEAT PRODS(R)	*	0-5 MO.	.002750	.007250	.011000
			6-11 MO.	.051750	.139500	.207000
			12-23 MO.	.075500	.129750	.302000
			2-65+ YR.	.196000	.325250	.784000

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
SODIUM ALUMINOSILICATE NAS 0178	28 IMIT DAIRY(R)	5	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	12.254620	20.132590	12.254620
			12-23 MO.	7.002640	29.761220	7.002640
			2-65+ YR.	7.877970	13.129950	7.877970
SODIUM ALUMINOSILICATE NAS 0178	48 SEAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	.053938	**********
			12-23 MO.	**********	.107876	**********
			2-65+ YR.	.053938	.269690	.053950
SODIUM ALUMINOSILICATE NAS 0178	ALL CATEGORIES ********** ********** **********	20	0-5 MO.	30.238810	45.830420	55.778010
			6-11 MO.	342.332980	907.238898	536.169230
			12-23 MO.	625.682910	1340.232206	1037.508220
			2-65+ YR.	1252.461748	2219.946280	2277.943220
SODIUM ALUMINUM PHOS NAS 0179	01 BAKED GOODS(R)	23	0-5 MO.	40.505900	53.610750	53.344640
			6-11 MO.	302.602900	617.119300	393.515840
			12-23 MO.	649.285750	1069.832300	855.083200
			2-65+ YR.	1634.532200	2427.971300	2152.613120
SODIUM ALUMINUM PHOS NAS 0179	03 OTHER GRAIN(R)	*	0-5 MO.	1.791400	6.090760	2.096250
			6-11 MO.	34.753160	102.468080	40.667250
			12-23 MO.	58.757920	135.788120	68.757000
			2-65+ YR.	99.601840	219.983920	116.551500
SODIUM ALUMINUM PHOS NAS 0179	06 CHEESE(R)	*	0-5 MO.	**********	2.217360	**********
			6-11 MO.	59.860720	215.083920	64.939860
			12-23 MO.	172.954080	492.253920	187.604040
			2-65+ YR.	208.431840	523.296960	226.086920
SODIUM ALUMINUM PHOS NAS 0179	10 MEAT PRODS(R)	*	0-5 MO.	1.493800	3.938200	1.493800
			6-11 MO.	28.110600	75.776400	28.110600
			12-23 MO.	41.011600	70.400200	41.011600
			2-65+ YR.	106.467200	176.675800	106.467200
SODIUM ALUMINUM PHOS NAS 0179	ALL CATEGORIES ********** ********** **********	27	0-5 MO.	43.791100	65.857070	56.934690
			6-11 MO.	425.335380	1010.447700	532.233550
			12-23 MO.	922.009350	1768.354540	1152.455840
			2-65+ YR.	2049.033080	3347.927980	2601.718740
SODIUM ASCORBATE NAS 0180	01 BAKED GOODS(R)	*	0-5 MO.	3.200080	4.235400	4.700160
			6-11 MO.	23.906480	48.754160	35.112960
			12-23 MO.	51.295400	84.519760	75.340800
			2-65+ YR.	129.132640	191.816560	189.665280

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
SODIUM ASCORBATE NAS 0180	02 BREAK CERLS(R)	8	0-5 MO.	.556320	1.576240	2.047500
			6-11 MO.	20.676560	55.446560	76.098750
			12-23 MO.	24.199920	47.194480	89.066250
			2-65+ YR.	18.544000	48.020960	68.250000
SODIUM ASCORBATE NAS 0180	05 MILK PRODS(R)	6	0-5 MO.	1.600560	1.185600	1.777140
			6-11 MO.	18.495360	88.940640	20.535240
			12-23 MO.	16.153800	51.692160	17.935950
			2-65+ YR.	11.707800	35.745840	12.939450
SODIUM ASCORBATE NAS 0180	08 PROCSD FRUT(R)	*	0-5 MO.	5.616500	15.057000	7.143530
			6-11 MO.	61.901000	154.155000	78.730820
			12-23 MO.	120.217000	238.641500	152.901940
			2-65+ YR.	141.368500	299.467000	179.804170
SODIUM ASCORBATE NAS 0180	10 MEAT PRODS(R)	14.	0-5 MO.	.363660	.958740	.543510
			6-11 MO.	6.843420	18.447480	10.227870
			12-23 MO.	9.984120	17.158140	14.921820
			2-65+ YR.	25.919040	43.011060	38.737440
SODIUM ASCORBATE NAS 0180	11 POULTRY(R)	*	0-5 MO.	.180000	.828000	.180000
			6-11 MO.	1.404000	4.752000	1.404000
			12-23 MO.	2.376000	6.624000	2.376000
			2-65+ YR.	4.644000	11.808000	4.644000
SODIUM ASCORBATE NAS 0180	13 FISH PRODS(R)	*	0-5 MO.	.036000	.108000	.036000
			6-11 MO.	.468000	1.764000	.468000
			12-23 MO.	1.944000	4.860000	1.944000
			2-65+ YR.	4.464000	11.124000	4.464000
SODIUM ASCORBATE NAS 0180	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
SODIUM ASCORBATE NAS 0180	24 BEV TYPE II(R)	10	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	**********	.000800	**********
			12-23 MO.	**********	.001600	**********
			2-65+ YR.	.260000	.755200	.520000
SODIUM ASCORBATE NAS 0180	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	1.575000	2.587500	1.575000
			12-23 MO.	.900000	3.825000	.900000
			2-65+ YR.	1.012500	1.687500	1.012500

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

	FOOD CATEGORY NO. NAME	# OF FIRMS	********** (AGE)	POSSIBLE DAILY INTAKE, MG. ********** AVERAGE	HIGH A	HIGH B
SODIUM ASCORBATE NAS 0180	30 HARD CANDY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.120000	.360000	.300000
			12-23 MO.	.360000	1.080000	.900000
			2-65+ YR.	.720000	2.040000	1.800000
SODIUM ASCORBATE NAS 0180	83 FORMULAS(R)	4	0-5 MO.	54.383400	99.630000	67.274280
			6-11 MO.	11.080300	52.795800	13.707360
			12-23 MO.	3.564000	1.004400	4.408800
SODIUM ASCORBATE NAS 0180	ALL CATEGORIES ********** ********** **********	41	0-5 MO.	65.936520	123.578980	83.702120
			6-11 MO.	146.470620	428.012940	238.160600
			12-23 MO.	230.994240	456.601040	360.695560
			2-65+ YR.	337.772480	645.484120	501.896840
SODIUM BENZOATE NAS 0181	01 BAKED GOODS(R)	19	0-5 MO.	1.692740	2.187450	3.728740
			6-11 MO.	12.346940	25.179980	27.640940
			12-23 MO.	26.492450	43.651780	59.737450
			2-65+ YR.	66.692920	99.067180	150.384920
SODIUM BENZOATE NAS 0181	02 BREAK CERLS(R)	*	0-5 MO.	.027900	.079050	.027900
			6-11 MO.	1.036950	2.780700	1.036950
			12-23 MO.	1.213850	2.368850	1.213850
			2-65+ YR.	.930000	2.408700	.930000
SODIUM BENZOATE NAS 0181	04 FATS OILS(R)	16	0-5 MO.	.485350	.485350	.489950
			6-11 MO.	2.717960	7.280250	2.743720
			12-23 MO.	6.115410	11.648400	6.173370
			2-65+ YR.	16.987250	30.674120	17.142250
SODIUM BENZOATE NAS 0181	05 MILK PRODS(R)	5	0-5 MO.	.996300	.738000	1.731780
			6-11 MO.	11.512800	55.368450	20.011080
			12-23 MO.	10.055250	32.176800	17.478150
			2-65+ YR.	7.287750	22.250700	12.667650
SODIUM BENZOATE NAS 0181	06 CHEESE(R)	5	0-5 MO.	**********	.000040	**********
			6-11 MO.	.001080	.003880	.001350
			12-23 MO.	.003120	.003880	.003900
			2-65+ YR.	.003760	.009440	.004700
SODIUM BENZOATE NAS 0181	07 FROZN DAIRY(R)	11	0-5 MO.	.115900	.475190	.154900
			6-11 MO.	1.101050	3.059760	1.471550
			12-23 MO.	1.668960	3.917420	2.230560
			2-65+ YR.	2.967040	7.151030	3.965440

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 A	HIGH B
SODIUM ERYTHORBATE NAS 0317	02 BREAK CERLS(R)	*	0-5 MO.	.012000	.034000	.045000
			6-11 MO.	.446000	1.196000	1.672500
			12-23 MO.	.522000	1.018000	1.957500
			2-65+ YR.	.400000	1.036000	1.500000
SODIUM ERYTHORBATE NAS 0317	05 MILK PRODS(R)	*	0-5 MO.	.004960	.003600	.012960
			6-11 MO.	.056160	.270090	.149760
			12-23 MO.	.049050	.156960	.130800
			2-65+ YR.	.035550	.108540	.054800
SODIUM ERYTHORBATE NAS 0317	10 MEAT PRODS(R)	17	0-5 MO.	.402930	1.062270	4.395340
			6-11 MO.	7.582410	20.439940	82.787580
			12-23 MO.	11.062260	19.010970	120.781880
			2-65+ YR.	28.717920	47.655630	313.552960
SODIUM ERYTHORBATE NAS 0317	11 POULTRY(R)	*	0-5 MO.	.062500	.237500	.062500
			6-11 MO.	.487500	1.650000	.487500
			12-23 MO.	.825000	2.200000	.825000
			2-65+ YR.	1.612500	4.100000	1.612500
SODIUM ERYTHORBATE NAS 0317	23 BEV TYPE I(R)	*	0-5 MO.	.008640	.012960	.008640
			6-11 MO.	.081720	.279720	.081720
			12-23 MO.	.195120	.585000	.195120
			2-65+ YR.	.374400	.999720	.374400
SODIUM ERYTHORBATE NAS 0317	24 BEV TYPE II(R)	20	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	**********	.001390	**********
			12-23 MO.	**********	.002700	**********
			2-65+ YR.	.451750	1.312160	.592500
SODIUM ERYTHORBATE NAS 0317	ALL CATEGORIES ********** ********** **********	37	0-5 MO.	.490930	1.400330	4.528440
			6-11 MO.	8.653790	23.836740	85.179060
			12-23 MO.	12.653430	23.073710	123.860300
			2-65+ YR.	31.592120	55.212050	317.687160
SODIUM FLUORIDE NAS 0318	23 BEV TYPE I(R)	*	0-5 MO.	.005280	.007920	.005280
			6-11 MO.	.049940	.170940	.049940
			12-23 MO.	.119240	.357500	.119240
			2-65+ YR.	.228800	.610940	.228800
SODIUM FLUORIDE NAS 0318	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.005280	.007920	.005280
			6-11 MO.	.049940	.170940	.049940
			12-23 MO.	.119240	.357500	.119240
			2-65+ YR.	.228800	.610940	.228800

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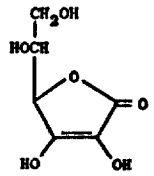
Ascorbic Acid. Vitamin C; L-ascorbic acid; L-xyloascorbic acid; 3-oxo-L-gulofuranolactone (enol form); L-3-ketothreohexuronic acid lactone; antiscorbutic vitamin; cevitamic acid; Cebione; Cantaxin; Cevalin; Cevatine; Cevimin; Cevitex; Cipca; Cebicure; C-Vimin; Cevitamin; Testascorbic; Allercorb; Cecon; Ce-Vi-Sol; Ascorin; Ascorteal; Cegiolan; Adenex; Ascorvit; Cevex; Lemascorb; Ciamin; Hybrin; Vitacee; Cantan; Catavin C; Celin; Cenetone; Cescorbat; Cereon; Cergona; Cetemican; Cetamid; Planavit C; Concemin; Scorbacid; Davitamon C; Proscorbin; Redoxon; Scorbu-C; Ribena; Vicelat; Vitacin; Vitacimin; Vitascorbol; Xitix; Cevitan; Laroscorbine. $C_6H_8O_6$; mol wt 176.12. C 40.91%, H 4.58%, O 54.51. Widely distributed in the plant and animal kingdom. Good sources are citrus fruits, hip berries, acerola, fresh tea leaves. Isolated from the adrenal cortex of ox and later from lemons and paprika (originally called hexuronic acid): Szent-Györgyi, *Biochem. J.* 22, 1387 (1928); Haworth, Szent-Györgyi, *Nature* 131, 24 (1933). Structure studies: Herbert *et al.*, *J. Chem. Soc.* 1933, 1270. Synthesis: Ault *et al.*, *ibid.* 1419; Reichstein *et al.*, *Helv. Chim. Acta* 16, 561, 1019 (1933); 17, 311, 510 (1934). Crystal structure: Hvoslef, *Acta Chem. Scand.* 18, 841 (1964). *Reviews:* Haworth, Hirst, *Ergeb. Vitamin u. Hormonforsch.* 2, 160 (1939); Rosenberg, *Chemistry and Physiology of the Vitamins*, Interscience, N.Y., 1945; Seitz, *Darstellung von Vitaminpräparaten*, Hirzel, Leipzig, 1939; PB Report 1676 (1946); Burns, in Kirk-Othmer, *Encyclopedia of Chemical Technology*, 2nd ed, New York, 1963; *Ann. N.Y. Acad. Sci.* 92, 1-332 (1961). The commercially available vitamin is produced by synthesis: One process in common use starts with D-glucose which is converted to D-sorbitol by hydrogenation. The D-sorbitol is made to yield L-sorbose by oxidation with *Acetobacter suboxydans*. Then a carboxyl group is introduced at C_1. This is done while the L-sorbose is in the form of its diacetone derivative. The resulting diacetone-2-keto-L-gulonic acid is then heated with HCl to give ascorbic acid. Alternate route from sorbose by oxidizing with nitrogen tetroxide: Gisvold, U.S. pat. 2,702,808 (1955 to U. of Minnesota). *See also* 2-Keto-L-gulonic acid.



Crystals (usually plates, sometimes needles, monoclinic system). Pleasant, sharp, acidic taste. Stable to air when dry. In impure prepns and in many natural products the vitamin oxidizes on exposure to air and light. d 1.65. mp 190-192° (some dec); $[\alpha]_D^{25}$ +20.5° to +21.5° (c = 1); $[\alpha]_D^{23}$ +48° (c = 1 in methanol). pH = 3 (5 mg/ml); pH = 2 (50 mg/ml); pK_1 = 4.17; pK_2 = 11.57. Absorption max 245 mμ (acid soln); 265 mμ (neutral soln). Redox potential of first stage at pH 5.0 is ϵ'_0 = +0.127 v. One gram dissolves in about 3 ml water, 30 ml alc, 50 ml absolute alc, 100 ml glycerol U.S.P., 20 ml propylene glycol. Solubility in hot water: 80.0% at 100°; 40.0% at 45°. Insoluble in ether, chloroform, benzene, petr ether, oils, fats, fat solvents. Possesses relatively strong reducing power, decolorizes many dyes. Aq solns are rapidly oxidized by air. The reaction is accelerated by alkalies, iron, copper. Forms stable metal salts, *see* Sodium Ascorbate. *Pharmaceutical Incompatibilities:* Vitamin C should not be formulated with sodium salicylate, sodium nitrite, theobromine sodium salicylate, methenamine. *Ref: C.A.* 46, 7707.

Calcium hypophosphite, $(C_6H_7O_6)CaH_2PO_2$, ***asphocalcium, Calscorbat.***

USE: Aside from its use as a vitamin, ascorbic acid or some derivatives, such as ascorbyl palmitate, are employed as antioxidants in foodstuffs, to prevent rancidity, to prevent the browning of cut apples and other fruit, in meat curing.

MED USE: Vitamin C deficiency. *Dose:* Oral 100 mg to 1 g.

VET USE: Has been reported favorable in certain cases of sterility of cattle and scurvy-like signs in dogs. *Dose* (used clinically and said to be effective where ascorbic acid deficiency exists): horses: 2-4 g s.c. repeated as needed; bulls: 1-2 g/450 kg body weight s.c. at 3- to 4-day intervals for 5 or 6 weeks; cattle: 1-2 g i.v. plus 2 g s.c. just before breeding; dogs: 25-75 mg s.c. or orally.

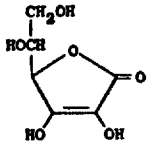
Note: One unit (U.S.P. or international) is the vitamin C activity of 0.05 mg of the U.S.P. ascorbic acid reference standard.

Calcium Ascorbate. *Ascorbic acid calcium salt.* $(C_6H_7O_6)_2Ca$; mol wt 390.32. $C_{12}H_{14}CaO_{12}$; C 39.93%, H 3.61%, Ca 10.27%, O 49.19%. Prepd from ascorbic acid and calcium carbonate by controlled precipitation in dil acetone or alcohol: Ruskin, Merrill, *Science* 105, 504 (1947); Ruskin, U.S. pat. 2,596,103 (1952); Ruskin, U.S. pat. 2,631,155 (1953 to Physiological Chemicals).

Dihydrate, $(C_6H_7O_6)_2Ca.2H_2O$, triclinic crystals, $[\alpha]_D^{20}$ +95.6° (c = 2.4). Freely sol in water; practically insol in methanol, ethanol. Aq solns are neutral and oxidize quickly. After prolonged storage calcium oxalate may precipitate. The addition of various stabilizers such as cysteine, has been patented.

MED USE: *See* Ascorbic Acid.

Isoascorbic Acid. D-Araboascorbic acid; erythorbic acid; isovitamin C; saccharosonic acid; glucosaccharonic acid; D-*erythro*-3-ketohexonic acid lactone; D-*erythro*-3-oxohexonic acid lactone; erycorbin; Mercate "5"; Neo-Cebicure. $C_6H_8O_6$; mol wt 176.12. C 40.91%, H 4.58%, O 54.51%. Epimer of L-ascorbic acid. Has one-twentieth of the vitamin C activity of L-ascorbic acid. Prepd by treating methyl 2-keto-D-gluconate with sodium methoxide: Maurer, Schiedt, *Ber.* 66, 1054 (1933); 67, 1239 (1934); Ohle *et al.*, *ibid.* 67, 324 (1934); Baird *et al.*, *J. Chem. Soc.* 1934, 63; Reichstein *et al.*, *Helv. Chim. Acta* 17, 516 (1934). Synthesis from sucrose: Heimann, Reiff, *Pharm. Zentralhalle* 93, 97 (1954). Production by *Penicillium* spp: Takahashi *et al.*, *Nature* 188, 411 (1960); U.S. pat. 3,052,609 (1962 to Sankyo); *Bull. Agr. Chem. Soc. Japan* 24, 533 (1960); *C.A.* 55, 1788 (1961). Conformation: Matsui *et al.*, *Agr. Biol. Chem.* (*Tokyo*) 27, 185 (1963).

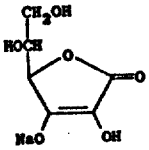


Shiny granular crystals from water or dioxane. Dec 174°. $[\alpha]_D^{18.5}$ −17° (c = 1.8 in 0.01*N* HCl); $[\alpha]_D^{20}$ −16.6° (H_2O). Soluble in water, alcohol, pyridine. Moderately sol in acetone. Slightly sol in glycerol.

Forms a sodium salt, ***sodium erythorbate, Mercate "20", Neo-Cebitate,*** crystals. Soluble in water. pH of aq solns of the sodium salt between 5 and 6. A 10% soln, made from commercial grade, may have a pH of 7.2 to 7.9. The free acid is more sol in water (40 g/100 ml H_2O) than the sodium salt (16 g/100 ml H_2O).

USE: Antioxidant for foods: Kadin, Osadca, *Agricult. & Food Chem.* 7, 358 (1959). *Human Toxicity:* Practically nontoxic.

Sodium Ascorbate. *Ascorbic acid sodium derivative;* vitamin C sodium; Ascorbin; Sodascorbate; Natrascorb; Cenolate; Ascorbicin; Cebitate. $C_6H_7NaO_6$; mol wt 198.12. C 36.38%, H 3.56%, Na 11.60%, O 48.46%, ascorbic acid 88.90%. One mg of the sodium salt is equivalent to 0.8890 mg of ascorbic acid, or one mg of the acid is equivalent to 1.1248 mg of sodium ascorbate. Prepn: Holland, U.S. pat. 2,442,005 (1948): Ascorbic acid is dissolved in water and an equivalent amount of sodium bicarbonate is added. After cessation of effervescence the sodium ascorbate is precipitated by the addn of isopropanol.



Minute crystals. Dec 218°. $[\alpha]_D^{20}$ +104.4° in H_2O. Freely sol in water at 25° = 62 g/100 ml H_2O. Even more sol in warm water (78 g/100 ml H_2O at 75°). pH of aq solns 5.6-7.0 or even higher. A 10% soln, made from a commercial grade, may have a pH of 7.4-7.7. Aq solns are unstable and subject to quick oxidation by air at pH above 6.0. Solns can be buffered with ascorbic acid solns which have a pH of 2.3-2.5.

USE: Aside from use in vitamin C prepns it can serve as antioxidant in chopped meat and other food, also in curing meat.

MED USE: In vitamin C deficiency.

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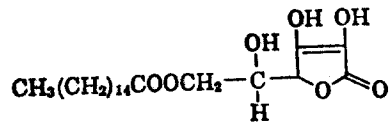
ASCORBYL PALMITATE

Palmitoyl L-Ascorbic Acid



$C_{22}H_{38}O_7$ Mol. wt. 414.54

DESCRIPTION

A white or yellowish white powder having a characteristic citrus-like odor. It is very slightly soluble in water and in vegetable oils. One gram dissolves in about 4.5 ml. of alcohol. A 1 in 10 solution in alcohol decolorizes a 1 in 1000 solution of 2,6-dichlorophenol-indophenol sodium.

SPECIFICATIONS

Assay. Not less than 95.0 percent of $C_{22}H_{38}O_7$ calculated on the dried basis.

Melting range. Between 107° and 117°.

Specific rotation, $[\alpha]_D^{25°}$. Between +21° and +24° calculated on the dried basis.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Loss on drying. Not more than 2 percent.

Residue on ignition. Not more than 0.1 percent.

TESTS

Assay. Dissolve about 300 mg., accurately weighed, in 50 ml. of alcohol in a 250-ml. Erlenmeyer flask, add 30 ml. of water, and immediately titrate with 0.1 *N* iodine to a yellow color which persists for at least 30 seconds. Each ml. of 0.1 *N* iodine is equivalent to 20.73 mg. of $C_{22}H_{38}O_7$.

Melting range. Determine as directed in *Procedure for Class Ia*, page 931.

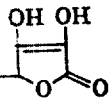
Specific rotation, page 939. Determine in a solution containing 1 gram in 10 ml. of methanol.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

ITATE

c Acid



Mol. wt. 414.54

ing a characteristic citrus-like
er and in vegetable oils. One
A 1 in 10 solution in alcohol
orophenol-indophenol sodium.

$H_{38}C$ alculated on the dried

21° and +24° calculated on

parts per million (0.0003 per-

an 10 parts per million (0.001

ercent.
n 0.1 percent.

urately weighed, in 50 ml. of
add 30 ml. of water, and im-
ellow color which persists for at
ne is equivalent to 20.73 mg. of

ced in *Procedure for Class Ia*,

mine in a solution containing 1

ed as directed for organic com-
rsenic Test, page 865.
2-gram sample as directed in
age 920, using 20 mcg. of lead ion

Loss on drying, page 931. Dry in a vacuum oven at 56° to 60° for 1 hour.

Residue on ignition. Ignite 2 grams as directed in the general method, page 945.

Packaging and storage. Store in tight containers, preferably in a cool, dry place.

Functional use in foods. Antioxidant.

DL-ASPARTIC ACID

DL-Aminosuccinic Acid

$HOOCCH_2CH(NH_2)COOH$

$C_4H_7NO_4$ Mol. wt. 133.10

DESCRIPTION

Colorless or white, odorless crystals having an acid taste. It is slightly soluble in water, but insoluble in alcohol and in ether. It is optically inactive and melts with decomposition at about 280°.

IDENTIFICATION

To 5 ml. of a 1 in 1000 solution add 1 ml. of triketohydrindene hydrate T.S. A bluish purple color is produced.

SPECIFICATIONS

Assay. Not less than 98.0 percent and not more than the equivalent of 102.0 percent of $C_4H_7NO_4$, calculated on the dried basis.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).
Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).
Lead. Not more than 10 parts per million (0.001 percent).
Loss on drying. Not more than 0.3 percent.
Residue on ignition. Not more than 0.1 percent.

TESTS

Assay. Dissolve about 250 mg., accurately weighed, in 100 ml. of recently boiled and cooled water, add phenolphthalein T.S., and titrate with 0.1 *N* sodium hydroxide to the first appearance of a faint pink color that persists for at least 30 seconds. Each ml. of 0.1 *N* sodium hydroxide is equivalent to 13.31 mg. of $C_4H_7NO_4$.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

owder. It is nearly odorless and
is soluble in alkaline solutions or
bine with the calcium. It is in-

0.1 *N* sodium hydroxide add 1 ml.
gelatinous precipitate is formed.
ed for *Test A* add 1 ml. of diluted
precipitate is formed.
uirements of *Identification Test C*

ginate with diluted hydrochloric
s positive tests for *Calcium*, page

not less than 18.0 percent and not
ide (CO_2) corresponding to be-
m r ate (Equiv. wt. 219.00).
he dried basis.

3 parts per million (0.0003 per-

than 40 parts per million (0.004

0.2 percent.
million (0.001 percent).
5 percent.

er *Alginates Assay*, page 863.
nsumed in the assay is equivalent
. wt. 219.00).
Ash in the monograph on *Alginic*

red as directed for organic com-
Arsenic Test, page 865.
a 500-mg. sample as directed in
est, page 920, but use a platinum
es not exceed that produced in a
of lead ion (Pb).
irected under *Insoluble matter* in
, but use 15 ml. of 0.1 *N* sodium

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Loss on drying, page 931. Dry at 105° for 4 hours.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Stabilizer; thickener; emulsifier.

CALCIUM ASCORBATE

$C_{12}H_{14}CaO_{12}.2H_2O$ **Mol. wt. 426.35**

DESCRIPTION

A white to slightly yellow, odorless, crystalline powder. It is soluble in water, slightly soluble in alcohol, and insoluble in ether. The pH of a 1 in 10 solution is between 6.8 and 7.4. A 1 in 10 solution gives positive tests for *Calcium*, page 926, and it decolorizes dichlorophenolindophenol T.S.

SPECIFICATIONS

Assay. Not less than 98.0 percent of $C_{12}H_{14}CaO_{12}.2H_2O$.

Specific rotation, $[\alpha]_D^{25°}$. Between +95° and +97°.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Fluoride. Not more than 10 parts per million (0.001 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Oxalate. Passes test.

TESTS

Assay. Dissolve about 300 mg., accurately weighed, in 50 ml. of water in a 250-ml. Erlenmeyer flask, and immediately titrate with 0.1 *N* iodine to a pale yellow color which persists for at least 30 seconds. Each ml. of 0.1 *N* iodine is equivalent to 10.66 mg. of $C_{12}H_{14}CaO_{12}\cdot 2H_2O$.

Specific rotation, page 939. Determine in a solution containing 1 gram in each 20 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Fluoride. Proceed as directed in the *Fluoride Limit Test*, page 917.

Heavy metals. A solution of 2 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Oxalate. To a solution of 1 gram in 10 ml. of water add 2 drops of glacial acetic acid and 5 ml. of a 1 in 10 solution of calcium acetate. The solution remains clear after standing for 5 minutes.

Packaging and storage. Store in tight containers, preferably in a cool, dry place.

Functional use in foods. Antioxidant.

CALCIUM BROMATE

$Ca(BrO_3)_2.H_2O$ **Mol. wt.** 313.90

DESCRIPTION

A white crystalline powder. It is very soluble in water.

IDENTIFICATION

A. A 1 in 20 solution in diluted hydrochloric acid T.S. imparts a transient yellowish red color to a nonluminous flame.

B. To a 1 in 20 solution add sulfurous acid dropwise. A yellow color is produced which disappears upon the addition of an excess of sulfurous acid.

SPECIFICATIONS

Assay. Not less than 99.8 percent of $Ca(BrO_3)_2.H_2O$.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 40 parts per million (0.004 percent).

Lead. Not more than 10 parts per million (0.001 percent).

TESTS

Assay. Dissolve about 900 mg., accurately weighed, in 50 ml. of water in a 250-ml. glass-stoppered Erlenmeyer flask. Add 3 grams of potassium iodide, followed by 3 ml. of hydrochloric acid. Allow the mixture to stand for 5 minutes, add 100 ml. of cold water, and titrate the liberated iodine with 0.1 *N* sodium thiosulfate, adding starch T.S. near the end-point. Perform a blank determination (see page 2[illegible]). Each ml. of 0.1 *N* sodium thiosulfate is equivalent to 26.16 mg. of $Ca(BrO_3)_2.H_2O$.

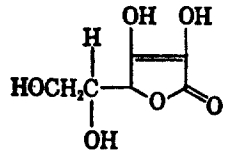
Arsenic. Dissolve 1 gram in a mixture of 5 ml. of hydrochloric acid and 5 ml. of water, and evaporate the solution until crystals appear. Cool, dissolve the residue in water, and dilute to 35 ml. This solution meets the requirements of the *Arsenic Test*, page 865.

Sample Solution for the Determination of Heavy Metals and Lead. Dissolve 2 grams in 10 ml. of water, add

Packaging and storage. Store in tight containers.
Functional use in foods. Flavoring agent.

ERYTHORBIC ACID

D-Araboascorbic Acid



$C_6H_8O_6$ Mol. wt. 176.13

DESCRIPTION

White or slightly yellow crystals or powder. On exposure to light it gradually darkens. In the dry state it is reasonably stable in air, but in solution it rapidly deteriorates in the presence of air. It melts between 164° and 171° with decomposition. One gram is soluble in about 2.5 ml. of water and in about 20 ml. of alcohol. It is slightly soluble in glycerin.

IDENTIFICATION

A. A 1 in 50 solution slowly reduces alkaline cupric tartrate T.S. at 25°, but more readily upon heating.

B. To 2 ml. of a 1 in 50 solution add a few drops of sodium nitroferricyanide T.S., followed by 1 ml. of approximately 0.1 *N* sodium hydroxide. A transient blue color is produced immediately.

C. Dissolve about 15 mg. in 15 ml. of a trichloroacetic acid solution (1 in 20), add about 200 mg. of activated charcoal, and shake the mixture vigorously for 1 minute. Filter through a small fluted filter, refiltering if necessary to obtain a clear filtrate. To 5 ml. of the clear filtrate add 1 drop of pyrrole, agitate the mixture until the pyrrole is dissolved, then heat in a water bath at 50°. A blue color develops.

SPECIFICATIONS

Assay. Not less than 99.0 percent of $C_6H_8O_6$.

Specific rotation, $[\alpha]_D^{25°}$. Between −16.5° and −18.0°.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Residue on ignition. Not more than 0.3 percent.

TESTS

Assay. Dissolve about 400 mg., accurately weighed, in a mixture of 100 ml. of water, recently boiled and cooled, and 25 ml. of diluted sulfuric acid T.S. Titrate the solution immediately with 0.1 *N* iodine, adding starch T.S. near the end-point. Each ml. of 0.1 *N* iodine is equivalent to 8.806 mg. of $C_6H_8O_6$.

Specific rotation. Transfer about 2.5 grams, accurately weighed, into a 25-ml. volumetric flask, dissolve it in about 20 ml. of water, and dilute to volume. Determine the specific rotation as directed under *Optical Rotation*, page 939.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 1 gram in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

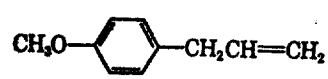
Residue on ignition, page 945. Ignite 1 gram as directed in the general method.

Packaging and storage. Store in tight, light-resistant containers.

Functional use in foods. Preservative; antioxidant.

ESTRAGOLE

p-Allylanisole



$C_{10}H_{12}O$ **Mol. wt. 148.21**

DESCRIPTION

A colorless to light yellow liquid having an anise-like odor. It is soluble in alcohol, but practically insoluble in water.

SPECIFICATIONS

Refractive index. Between 1.517 and 1.522 at 20°.

Solubility in alcohol. Passes test.

Specific gravity. Between 0.957 and 0.965.

9'a

PHOSPHATE, BASIC

sal

ed of an autogenous mixture of an ate [approximately $Na_8Al_2(OH)_2$- sic sodium phosphate. It is solu- n phosphate moiety is soluble in n phosphate moiety is only spar- olution in dilute hydrochloric acid uminum, page 925, and for *Phos-* ne flame test for *Sodium*, page 928.

nd not more than 12.5 percent of

9 percent.

n 3 parts per million (0.0003 per-

ts per million (0.0025 percent).
e th 40 parts per million (0.004

er million (0.001 percent).

s, accurately weighed, into a 250- hydrochloric acid and one glass utes. Cool, dilute to volume with this solution to a 250-ml. beaker, ralize with ammonia T.S. Add til the precipitate just dissolves, heat to 70°–80°. Add 10 ml. of nt ammonium acetate T.S. until 30 ml. in excess. Digest at 70° for ously dried and weighed Gooch hot water. Dry at 105° for 2 the precipitate so obtained corre-

to 800° for 2 hours.
10 ml. of dilute hydrochloric acid e *Arsenic Test*, page 865.
grams, and proceed as directed in

Heavy metals. Dissolve 500 mg. in 2.5 ml. of diluted hydrochloric acid T.S., and add water to make 25 ml. This solution meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A solution of 1 gram in 5 ml. of diluted hydrochloric acid T.S. meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Emulsifier.

SODIUM ASCORBATE

Vitamin C Sodium; Sodium L-Ascorbate

$C_6H_7NaO_6$ Mol. wt. 198.11

DESCRIPTION

A white or almost white crystalline solid. One gram is soluble in 2 ml. of water. The pH of a 1 in 10 solution is about 7.5.

IDENTIFICATION

A. A 1 in 50 solution slowly reduces alkaline cupric tartrate T.S. at 25°, but more readily upon heating.

B. To 2 ml. of a 1 in 50 solution of the sample acidified with 0.5 ml. of 0.1 *N* hydrochloric acid add 4 drops of methylene blue T.S., and warm to 40°. The deep blue color is practically discharged within 3 minutes.

C. Dissolve 15 mg. of the sample in 15 ml. of a 1 in 20 solution of trichloroacetic acid, add about 200 mg. of activated charcoal, shake vigorously for 1 minute, and filter through a small fluted filter, returning the filtrate, if necessary, until clear. To 5 ml. of the filtrate add 1 drop of pyrrole, and agitate gently until dissolved, and then heat in a water bath at 50°. A blue color develops.

D. It gives positive tests for *Sodium*, page 928.

SPECIFICATIONS

Assay. Not less than 99.0 percent and not more than the equivalent of 101.0 percent of $C_6H_7NaO_6$ after drying.

Specific rotation, $[\alpha]_D^{25°}$. Between +103° and +108°.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).
Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).
Lead. Not more than 10 parts per million (0.001 percent).
Loss on drying. Not more than 0.25 percent.

TESTS

Assay. Dissolve about 400 mg., previously dried over phosphorus pentoxide for 24 hours and accurately weighed, in a mixture of 100 ml. of water, recently boiled and cooled, and 25 ml. of diluted sulfuric acid T.S., and titrate with 0.1 *N* iodine, adding starch T.S. near the end-point. Each ml. of 0.1 *N* iodine is equivalent to 9.905 mg. of $C_6H_7NaO_6$.

Specific rotation, page 939. Determine in a solution containing 1 gram in each 10 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 1-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Loss on drying, page 931. Dry over phosphorus pentoxide for 24 hours.

Packaging and storage. Store in tight, light-resistant containers.

Functional use in foods. Antioxidant; nutrient; dietary supplement.

SODIUM BENZOATE

$C_7H_5NaO_2$ **Mol. wt. 144.11**

DECRIPTION

White, odorless or nearly odorless, granules, crystalline powder or flakes. One gram dissolves in 2 ml. of water, in 75 ml. of alcohol, and in 50 ml. of 90 percent alcohol. It gives positive tests for *Sodium*, page 928, and for *Benzoate*, page 926.

SPECIFICATIONS

Assay. Not less than 99.0 percent of $C_7H_5NaO_2$, calculated on the dried basis.

Limits of Impurities

Alkalinity (as NaOH). Not more than 0.04 percent.

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Water. Not more than 1.5 percent.

.TE

state

.xH_2O

.. wt. (anhydrous) 142.09

:d of sodium acetate and
vstalline solid having an
:n about 1 ml. of water.
:d 5.0. A 1 in 10 solution
:or *Sodium*, page 928.

more than 41.0 percent of
than 58.0 percent and not
CH_3COONa).

illion (0.0003 per-

:0 parts per million (0.001

:ormic acid). Not more

: 4 grams, dissolve it in 50
:d titrate with 1*N* sodium
:ide is equivalent to 60.05

:y about 500 mg., dissolve
with 0.1 *N* perchloric acid,
:. Each ml. of 0.1 *N* per-
C_2H_3COONa.
:rimetric Method, page 977.
of water meets the require-

. 25 ml. of water meets the
: 920, using 20 mcg. of lead

olve 1.0 gram in about 50
:id T.S., and heat the solu-

tion to between 80° and 90°. Titrate the hot solution with 0.1 *N* potassium permanganate to a faint pink color that persists for at least 15 seconds. Each ml. of 0.1 *N* potassium permanganate is equivalent to 2.301 mg. of CH_2O_2.

Packaging and storage. Store in tight containers.

Functional use in foods. Sequestrant; preservative; mold and rope inhibitor.

SODIUM ERYTHORBATE

$C_6H_7NaO_6 . H_2O$ Mol. wt. 216.12

DESCRIPTION

White, odorless, crystalline powder or granules. In the dry state it is reasonably stable in air, but in solution it deteriorates in the presence of air, trace metals, heat, and light. One gram dissolves in about 7 ml. of water. The pH of a 1 in 20 solution is between 5.5 and 8.0.

IDENTIFICATION

A. A 1 in 50 solution slowly reduces alkaline cupric tartrate T.S. at 25°, but more readily upon heating.

B. To 2 ml. of a 1 in 50 solution acidified with 0.5 ml. of 0.1 *N* hydrochloric acid add a few drops of sodium nitroferricyanide T.S., followed by 1 ml. of 0.1 *N* sodium hydroxide. A transient blue color is produced immediately.

C. It gives positive tests for *Sodium*, page 928.

SPECIFICATIONS

Assay. Not less than 98.0 percent of $C_6H_7NaO_6 . H_2O$.

Specific rotation, $[\alpha]_D^{25°}$. Between +95.5° and +98.0°.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 30 parts per million (0.003 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Oxalate. Passes test.

TESTS

Assay. Dissolve about 400 mg., accurately weighed, in a mixture of 100 ml. of water, recently boiled and cooled, and 25 ml. of diluted sulfuric acid T.S., and immediately titrate with 0.1 *N* iodine, adding starch T.S. near the end-point. Each ml. of 0.1 *N* iodine is equivalent to 10.81 mg. of $C_6H_7NaO_6 . H_2O$.

Specific rotation, page 939. Determine in a solution containing 1 gram in each 10 ml.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 670-mg. sample as directed in *Method II* under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Oxalate. To a solution of 1 gram in 10 ml. of water add 2 drops of glacial acetic acid and 5 ml. of a 1 in 10 solution of calcium acetate. The solution remains clear.

Packaging and storage. Store in tight, light-resistant containers.

Functional use in foods. Preservative; antioxidant.

SODIUM FERRIC PYROPHOSPHATE

Sodium Iron Pyrophosphate

$Na_8Fe_4(P_2O_7)_5 . xH_2O$ Mol. wt. (anhydrous) 1277.00

DESCRIPTION

A white to tan odorless powder. It is insoluble in water but is soluble in hydrochloric acid.

IDENTIFICATION

Dissolve 500 mg. in 5 ml. of dilute hydrochloric acid (1 in 2), and add an excess of sodium hydroxide T.S. A reddish brown precipitate forms. Age the solution for several minutes, and then filter, discarding the first few ml. To 5 ml. of the clear filtrate add 1 drop of bromophenol blue T.S., and titrate with 1 *N* hydrochloric acid to a green color. Add 10 ml. of a 1 in 8 solution of zinc sulfate, and readjust the pH to 3.8 (green color). A white precipitate forms (distinction from *orthophosphates*).

SPECIFICATIONS

Assay. Not less than 14.5 percent and not more than 16.0 percent of Fe.

Loss on ignition. Not more than 8 percent.

Limits of Impurities

Fluoride. Not more than 50 parts per million (0.005 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Mercury. Not more than 3 parts per million (0.0003 percent).

125a

ASCORBYL PALMITATE
CHAS. PFIZER & CO., INC., Chemical Division.

ASPARAGINE
AJINOMOTO CO. OF NEW YORK, INC.
AMERICAN CYANAMID CO., Fine Chemicals Dept.
EASTERN CHEMICAL CORP.
Inland Alkaloid Co.
Pfanstiehl Laboratories, Inc.

dl-ASPARTIC ACID AND SALTS
Degussa Inc., Chemicals Division.
HEXAGON LABORATORIES, INC.
Nutritional Biochemicals Corp.
POLYCHEMICAL LABORATORIES, INC.

1-ASPARTIC ACID AND SALTS
AJINOMOTO CO. OF NEW YORK, INC.
HENLEY & CO., INC.
POLYCHEMICAL LABORATORIES, INC.,
S.S.T. CORP.

ASPHALT
Allied Asphalt & Mineral Corp.
AMERICAN GILSONITE CO.
Atlantic Richfield Co., Products Division.
Crowley Tar Products Co., Inc.
HUMBLE OIL & REFINING CO.
HUMMEL CHEMICAL CO., INC.
INDUSTRIAL RAW MATERIALS CORP.
Leonard Refineries, Inc.
Marathon Oil Co., Special Products.
Melanol Corp.
Mobil Oil Corp., Special Products Dept.
SUN OIL CO., DX Division.
WITCO CHEMICAL CORP., Pioneer Division.
ZIEGLER CHEMICAL & MINERAL CORP.
Zophar Mills, Inc.

ASPHALT COMPOUNDS
Allied Asphalt & Mineral Corp.
Melanol Corp.
National Research & Chemical Co.
George W. Whitesides Co., Inc.
WITCO CHEMICAL CORP., Pioneer Division.
ZIEGLER CHEMICAL & MINERAL CORP.
Zophar Mills, Inc.

ASPHALT CUTBACKS
Allied Asphalt & Mineral Corp.
HUMBLE OIL & REFINING CO.
INDUSTRIAL RAW MATERIALS CORP.
Leonard Refineries, Inc.
Melanol Corp.
Mobil Oil Corp., Special Products Dept.
George W. Whitesides Co., Inc.
WITCO CHEMICAL CORP., Golden Bear Division.
WITCO CHEMICAL CORP., Pioneer Division.
ZIEGLER CHEMICAL & MINERAL CORP.
Zophar Mills, Inc.

ASPHALT EMULSIONS
Melanol Corp.
WITCO CHEMICAL CORP., Golden Bear Division.
WITCO CHEMICAL CORP., Pioneer Division.

For Addresses of Suppliers—See Part II

ARYL MERCAPTANS See Aromatic Mercaptans

ARYLIDES
AMERICAN HOECHST CORP., Organic Intermediates Dept.

ASAFETIDA
MEER CORP.
S. B. PENICK & CO.

ASBESTOS
AMERICAN SMELTING & REFINING CO.
Buffalo Solvents & Chemicals Corp.
Carey-Canadian Mines, Ltd.
Commercial Chemicals, Inc.
Filter-Media Co.
Jensen-Souders & Associates, Inc.
Johns-Manville Products Corp., Celite Division.
McKESSON CHEMICAL CO.
Geo. A. Rowley & Co., Inc.
THOMPSON-HAYWARD CHEMICAL CO.
Charles A. Wagner Co., Inc.
WHITTAKER, CLARK & DANIELS, INC.

ASBESTOS FIBERS
Carey-Canadian Mines, Ltd.
Chemicals & Pigments Corp.
Huxley Development Corp.
Johns-Manville Products Corp., Celite Division.
KRAFT CHEMICAL CO.
Lake Asbestos of Quebec, Ltd.
National Gypsum Co.
THOMPSON-HAYWARD CHEMICAL CO.

ASBESTOS FILLER
Carey-Canadian Mines, Ltd.
Huxley Development Corp.
Johns-Manville Products Corp., Celite Division.

ASBESTOS PACKING
Johns-Manville Products Corp., Celite Division.

ASBESTOS PAPER
Johns-Manville Products Corp., Celite Division.

1-ASCORBIC ACID
AMERICAN ROLAND CORP.
DESMO CHEMICAL CORP.
DIAMOND SHAMROCK CHEMICAL CO., Fine Chemicals Division.
FALLEK PRODUCTS CO., INC.
Gymia Laboratories of America, Inc.
HELM NEW YORK CHEMICAL CORP.
HOFFMANN-LA ROCHE, INC., Roche Chemical Division.
HENRY INTERDONATI, INC.
INTERNATIONAL CHEMICAL CORP.
JERDAN CHEMICAL CORP.
MERCK & CO., INC., Merck Chemical Division.
CHAS. PFIZER & CO., INC., Chemical Division.
H. REISMAN CORP.
S. S. T. CORP.
SHEPARD CHEMICAL INDUSTRIES, INC.
THOMPSON-HAYWARD CHEMICAL CO.
GEORGE UHE CO., INC.
VGF CHEMICAL CORP.

For Addresses of Suppliers—See Part II

CALAMINE
Harrisons & Crosfield (America) Inc.
MALLINCKRODT CHEMICAL WORKS.
S. B. PENICK & CO.

CALAMUS OIL
CHARABOT & CO., INC.
GENTRY CORP., Aromatic Products Division.
S. B. PENICK & CO.

CALAMUS ROOT
Herbarium, Inc.

CALCIFEROL See Ergocalciferol

CALCIUM ACETATE
ACETO CHEMICAL CO., INC.
ALLIED CHEMICAL CORP., Specialty Chemicals Division.
BARIUM AND CHEMICALS, INC.
FALLEK PRODUCTS CO., INC.
MALLINCKRODT CHEMICAL WORKS.
MALLINCKRODT CHEMICAL WORKS, Washine Division.
MILLMASTER CHEMICAL CO.
PRIOR CHEMICAL CORP.
Progil, Inc.
SHEPHERD CHEMICAL CO.
GEORGE UHE CO., INC.

CALCIUM ACID PHOSPHATE See Calcium Phosphate, Monobasic.

CALCIUM ALUMINATE CEMENT
ALUMINUM CO. OF AMERICA

CALCIUM p-AMINOBENZOATE
GANE'S CHEMICAL WORKS, INC.

CALCIUM p-AMINOSALICYLATE
Academy Chemical Corp.
AMERICAN ROLAND CORP.
Consolidated Midland Corp., Chemical Division.
ROUSSEL CORP.

CALCIUM AMMONIUM NITRATE
H. J. Baker & Bro., Inc.

CALCIUM ARSENATE
Los Angeles Chemical Co.
RHODIA, INC., Chipman Division.
Woolfolk Chemical Works, Ltd.

CALCIUM ASCORBATE
S. B. PENICK & CO.
CHAS. PFIZER & CO., INC., Chemical Division.

CALCIUM BENZOATE
AMEND DRUG & CHEMICAL CO., INC.
City Chemical Corp.

CALCIUM BORATE
FINE ORGANICS, INC.

For Addresses of Suppliers—See Part II

ISOAMYL BENZYL ETHER
GIVAUDAN CORP.

ISOAMYL BROMIDE
ELANCO PRODUCTS CO., Industrial Chemicals Division.
STAUFFER CHEMICAL CO., Specialty Chemical Division, Benzol Products Dept.
WHITE CHEMICAL CORP.

ISOAMYL BUTYRATE
GIVAUDAN CORP.
WHITE CHEMICAL CORP.

ISOAMYL FORMATE
F. RITTER & CO.

ISOASCORBIC ACID
S. B. PENICK & CO.

ISOBORNEOL
BIDDLE SAWYER CORP.
De Laire, Inc.
RHODIA, INC., Aromatics Division.

ISOBORNYL ACETATE
AMERICAN HOECHST CORP., Chemicals & Plastics Division.
GIVAUDAN CORP.
NORDA ESSENTIAL OIL & CHEMICAL CO., INC.
ORBIS PRODUCTS CORP.
S. B. PENICK & CO.
RHODIA, INC., Aromatics Division.
UOP FRAGRANCES U.S.

ISOBORNYL PROPIONATE
GIVAUDAN CORP.
UOP FRAGRANCES U.S.

ISOBORNYL THIOCYANOACETATE
Chemical Insecticide Corp.
HALBY CHEMICAL CO., INC.

ISOBUTANE
AIR PRODUCTS & CHEMICALS, INC.
Marathon Oil Co., Special Products.
Matheson Gas Products.
PHILLIPS PETROLEUM CO., Special Products Division.
Precision Gas Products, Inc.
Technical Petroleum Co.
Tenneco Oil Co.

ISOBUTYL ACETATE
AMERICAN HOECHST CORP., Chemicals & Plastics Division.
AMERICAN MINERAL SPIRITS CO.
ASHLAND CHEMICAL CO., Industrial Chemicals & Solvents Division.
BELL CHEMICAL CO., INC.
CPS CHEMICAL.
CELANESE CHEMICAL CO.
DeMert & Dougherty, Inc.
EASTMAN CHEMICAL PRODUCTS, INC.

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SODIUM ASCORBATE—Continued
MERCK & CO., INC., Merck Chemical Division.
1262 CHAS. PFIZER & CO., INC., Chemical Division.
H. REISMAN CORP.
S. S. T. CORP.
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SODIUM AZIDE
BAIRD CHEMICAL INDUSTRIES, INC.
FAIRMOUNT CHEMICAL CO., INC.
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S. S. T. CORP.

SODIUM BARBITURATE
Abbott Laboratories, Chemical Marketing Division.

SODIUM BENZENE SULFONATE
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A. I. D. CHEMICAL CO., INC.
Abbott Laboratories, Chemical Marketing Division.
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l-Ascorbic and *d*-Isoascorbic Acids: Quantitative Separation and Assay

By CORA E. WEEKS and MIKE J. DEUTSCH (Division of Nutrition, Food and Drug Administration, Washington, D.C. 20204)

d-Isoascorbic (erythorbic) acid is a permissible food additive. Chemical assays do not differentiate between the additive and the vitamin, *l*-ascorbic acid. Chromatography on silicic acid-impregnated glass fiber paper separates the two acids in 25 minutes. They are extracted from the chromatogram and assayed by the microfluorometric method (Deutsch and Weeks, *This Journal*, *48*, 1248–1256 (1965)). Modifications have been developed for assaying small volumes of solution. Concentrations as low as 1.8 μg/20 μl can be successfully separated and assayed.

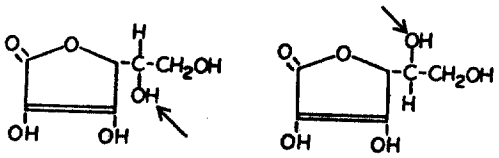
d-Isoascorbic acid, also referred to as erythorbic[1] or D-araboascorbic acid, is authorized for use in foods as a preservative (1). This compound prevents enzymatic browning in fruit tissues (2), flavor deterioration, and other changes associated with a rise in oxidation-reduction potential (3).

d-Isoascorbic acid and *l*-ascorbic acid are diastereoisomers (Fig. 1). The only difference in configuration is the relative position of the hydroxyl group on the fifth carbon. The general rule is that antiscorbutic activity depends on the presence of a five-membered lactone ring containing an enediol. This ring must lie on the right of the carbon chain when the formula is written according to the Fischer convention. *d*-Isoascorbic acid is antiscorbutic, but the activity is only about one-twentieth that of the *l*-form (4). Most analytical methods for *l*-ascorbic acid are based on the properties of the enediol. Since the enediol is common to both compounds, they give the same reaction. Because the quantities of *d*-isoascorbic acid suggested for antioxidant use are large enough to seriously influence vitamin assay results, it is essential to differentiate the two forms when assaying fabricated or processed foods.

[1] *d*-Isoascorbic acid must be designated as erythorbic acid when it is an ingredient of fabricated foods (Anon., *Federal Register* 23, 5873-4 (1958); 21 *CFR* Chap. 1, Part 3A, Sect. 3.51).

Published paper chromatographic data for these isomers, shown in Table 1, are in accord with the general chromatographic behavior of sugars. The ease with which carbohydrates can be separated depends more on configuration than on molecular size or number of hydrophilic groups (11). R_f values for simple hexoses and pentoses are inversely proportional to melting point (12); *l*-ascorbic acid, which melts at a higher temperature than its isomer, is less mobile. In the chromatography of sugars, the amount of water in the mobile solvent is critical (12). The data in Table 1 are arranged in order of increasing water content. The difference between R_f values for these acids increases directly with the amount of water in the organic phase, except for water-saturated phenol. Anomalous R_f values obtained with this solvent have been attributed to the formation of soluble compounds with sugars (12).

Assay of chromatographically separated *l*-ascorbic and *d*-isoascorbic acids has been reported for standard solutions (11), biological materials (5, 9), and foods (13) by the spectrophotometric dichloroindophenol (5, 9) and 2,4-dinitrophenylhydrazine (10, 13) methods. Development periods, including equilibration, range from 4.5 to 16 hours. Recoveries of the isomers added to fruit and vegetable juices ranged from 86 to 114% of *l*-ascorbic acid and from 65 to 81% of *d*-isoascorbic acid. Use of a high vacuum



l-ASCORBIC ACID

d-ISOASCORBIC ACID (syn. Erythorbic Acid, *d*-Araboascorbic Acid)

Fig. 1—Structural formulas of *l*-ascorbic and *d*-isoascorbic acids.

Table 1. Published paper chromatographic data for l-ascorbic and d-isoascorbic acids

Paper Used	Mobile Solvent	Paper Treatment[a]	Development Time,[b] hr	$R_f \times 100$ l-Ascorbic	$R_f \times 100$ d-Isoascorbic	Ref.
1	H_2O-satd collidine			40	41	(5)
2	H_2O-satd phenol + 1% HOAc			35	40	(5)
3	H_2O-satd phenol + $(COOH)_2$	2% $(COOH)_2$	20	43	50	(6)
4	BuOH:HOAC:H_2O (4:1:5)			37	38	(5)
5	BuOH:HOAC:H_2O (4:1:5)			42	43	(7)
6	H_2O-satd BuOH + $(COOH)_2$	2% $(COOH)_2$	16	29	30	(6)
7	CH_3CN:Me_2CO:H_2O:HOAc (80:5:15:1)[c]	1% HPO_3	5.5	34-40[d]	40-45[d]	(8)
				33-37[e]	41-45[e]	
				36-39[f]	41-45[f]	
				35-37[g]	42-45[g]	
8	CH_3CN:Me_2CO:H_2O:HOAc (80:5:15:1.2)	2% HPO_3	3	38	43	(9)
9	H_2O-satd MeCOEt	3% HPO_3	5-6	43	53	(10)
		3% HPO_3-3% glycerol	5-6	40	49	(10)

[a] Whatman No. 1 papers were used for 1-4, 6, and 8; Whatman No. 1 or 4 for 7; and Toyo Filter Paper No. 50 or 51 for 9.
[b] Not including equilibration.
[c] R_f values are a range of four determinations.
[d] 0.01*M* isomer applied individually.
[e] 0.01*M* of both isomers applied in mixture.
[f] 0.1*M* isomer applied individually.
[g] 0.1*M* of both isomers applied in mixture.

induction heating apparatus and acetonitrile-acetone-10% metaphosphoric acid as mobile solvent gives results within ± 5% after correction for a 5% loss (13). Data obtained on foods by this technique were not reported.

It was our purpose to develop a rapid separation and feasible assay for these isomers at their extreme dilution in foods.

Experimental

Silicic acid-impregnated glass fiber chromatography has been used to separate sugars (14). In the procedure described, the glass fiber paper with acidic properties is further treated with metaphosphoric acid-glycerol solution. Metaphosphoric acid stabilizes the isomers (8), and polyhydric alcohols also have a stabilizing effect (15), as well as serving as partition liquids (16). The mobile solvent is water-saturated methyl ethyl ketone. No equilibration period is necessary. The isomers are separated in 25 minutes by ascending chromatography; R_f is 0.52 for *l*-ascorbic acid and 0.63 for *d*-isoascorbic acid. A concentration of each isomer twelve times that of the other was easily separable. Although the manufacturer of the paper suggests a sample size of 0.1 to 2.0 μg, no severe tailing was encountered at higher concentrations.

A chromatogram of fruit beverages, to which *d*-isoascorbic acid was added, is shown in Fig. 2. The samples were extracted with a metaphosphoric acid-acetic acid solution before chromatography. Phosphomolybdic

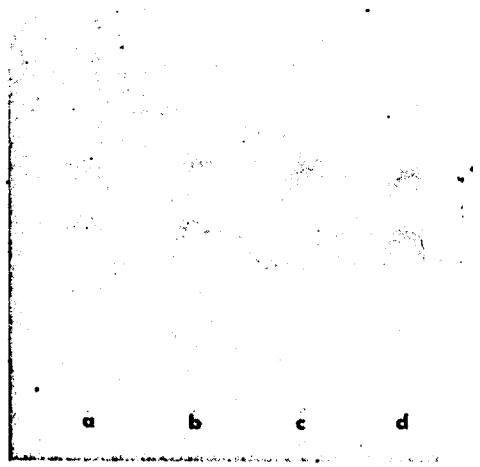


Fig. 2—Chromatogram of fruit beverages with added *d*-isoascorbic acid on silicic acid-impregnated glass fiber paper; *a*, standard mixture; *b*, sweetened pineapple juice; *c*, mango nectar consisting of pulp in sugar sirup (without *l*-ascorbic acid); and *d*, blend of apple, apricot, orange, pineapple, and prune juices with added sugar, gum arabic, and tragacanth, as well as other excipients.

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oascorbic acids

$R_f \times 100$		
As-corbic	d-Isoas-corbic	Ref.
40	41	(5)
35	40	(5)
43	50	(6)
37	38	(5)
42	43	(7)
29	30	(6)
34 40[d]	40–45[d]	(8)
33 37[e]	41 45[e]	
36 39[f]	41–45[f]	
35–37[g]	42 45[g]	
38	43	(9)
43	53	(10)
40	49	(10)

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fruit beverages, to
was added, is shown
were extracted with
acetic acid solution
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c d

: beverages with added
acid-impregnated glass
ire; b, sweetened pine-
consisting of pulp in
ic acid); and d, blend
pineapple, and prune
m arabic, and traga-
er excipients.

acid was used to detect the isomers, which appear as blue spots. This chromogenic reagent is suitable for screening (qualitative evaluation) because the spots are visible longer than on the chromatogram developed with dichloroindophenol. The amounts of *l*-form and *d*-iso-form were about 5 and 4 μg, respectively.

The blend sample (Spot d) was artificially colored. Natural cranberry red forms two crescents slightly above the origin. Concord grape anthrocyanins streak with the movement of the solvent front. Even in samples containing much cellular material, depectinization or other clarification procedures are not necessary. A large amount of natural and added constituents does not hinder the ease with which the isomers separate.

In the assay, the chromatogram is sprayed with dichloroindophenol solution, and the acids appear as white spots on a pink background. The spots and blanks are removed by cutting disks of equal size from the chromatogram. The acids are eluted by immersing the disks in 2 ml metaphosphoric acid-acetic acid solution and assayed by the microfluorometric method (17). Because of the small volume of assay solution, the oxidation step had to be modified and the concentrations of reagents adjusted.

Dichloroindophenol, rather than Norit, is used for oxidation. A two-fold excess of this reagent produces a yellow substance in the presence of *o*-phenylenediamine. Fluorescence values are diminished about 5%. The yellow product absorption maximum is at 450 mμ and the quinoxaline fluorescence maximum is at 460 mμ. Reduction in fluorescence readings is probably due to the "inner filter" effect described by Udenfriend (18). This interference is avoided by extracting excess dichloroindophenol with xylene before adding the diamine solution.

The concentration of *o*-phenylenediamine solution is one-half that used in the previously published microfluorometric method because of the low concentration of the isomers.

Standard solutions containing from 1.8 to 6.0 μg of each isomer in equal amount/20 μl spot were chromatographed and assayed. Six determinations were made at the 1.8 and 6.0 μg levels and seven at the other two levels. The plots of average points and regression equations are shown in Fig. 3. In the equation, y = fluorescence readings corrected by subtracting the paper blanks, and x = μg/20 μl of chromatographed solution. The standard deviation of the slope was ± 0.16 μg for *l*-ascorbic acid and ± 0.24 μg for *d*-isoascorbic acid. Greater variation for *d*-isoascorbic acid may be due to its slightly greater oxidation rate.

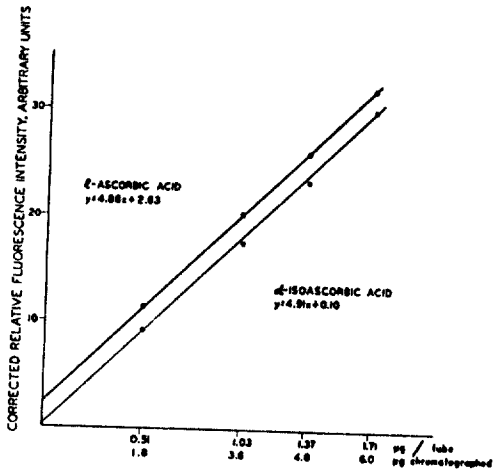

Fig. 3—Fluorescence reading vs concentration of chromatographed *l*-ascorbic and *d*-isoascorbic acids.

METHOD[2]

Use (e)(*1*),(2) and (g)-(k) listed under **Reagents and Apparatus** in (17) and the following:

Apparatus

(a) *Chromatographic tank.*—Metal, with trough, rods, and cover (No. 3106-F05, A. H. Thomas, Philadelphia, Pa.).

(b) *Chromatographic sprayer.*—Such as No. 3118-J (A. H. Thomas) or No. K-42250 (Kontes Glass Co., Vineland, N.J.).

Reagents

(a) *Treated silicic acid-impregnated glass fiber paper.*—Immerse 7 × 8" sheets of silicic acid-impregnated glass fiber paper with acidic

[2] The following reagents and apparatus have been found satisfactory; other commercial sources may be equally satisfactory.

properties (No. GFP-3, Applied Science Laboratories, Inc., P.O. Box 440, State College, Pa. 16801) in 3% (w/v) metaphosphoric acid-3% (v/v) glycerol solution for 15 min. Suspend in draft of hood and air-dry overnight. Use within 24 hr.

(b) *Mobile solvent.*—Water-saturated methyl ethyl ketone (No. M-208, Fisher Scientific Co.). Prepare 24 hr before use. Store in amber bottle.

(c) *Standard solutions.*—Prepare fresh for each assay. (*1*) *Ascorbic acid standard:* Accurately weigh USP Reference Standard Ascorbic Acid (USP Reference Standards, 46 Park Ave., New York) that has been stored in a desiccator away from direct sunlight. Dilute to volume immediately before use with metaphosphoric acid-acetic acid extracting solution ((e)(*1*) in (17)) so that each 20 μl contains about 3.6–20 μg ascorbic acid. Dilute an aliquot of this solution with extracting solution so that each 20 μl contains 1.8–10 μg ascorbic acid. (This standard solution should be about the same concentration as the sample.) (*2*) *d-Isoascorbic acid standard solution:* Accurately weigh *d*-isoascorbic acid (Nutritional Biochemicals Corp., 21010 Miles Ave., Cleveland, Ohio 44128) that has been stored in a desiccator away from direct sunlight. Proceed as in (c)(*1*), beginning, "Dilute to volume. . ." . (*3*) *Ascorbic-d-isoascorbic acids standard solution:* Proceed as in (c)(*1*) and (*2*) through ". . . each 20 μl contains about 3.6–20 μg ascorbic acid." Combine an aliquot of each of these solutions and dilute so that each 20 μl contains 1.8–10 μg ascorbic acid and 1.8–10 μg *d*-isoascorbic acid. (The concentration of each acid in this solution should be about the same concentration as the sample.)

(d) *2,6-Dichloroindophenol solution.*—Dissolve 29 mg 2,6-dichloroindophenol sodium salt (No. 3463, Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y. 14603) in 100 ml water. Heat, if necessary, to not more than 37°. Filter through Whatman No. 12 paper. Store in nonactinic glass-stoppered bottle in refrigerator. Use within one week. (*1*) *Chromogenic reagent:* Dilute an aliquot of 2,6-dichloroindophenol solution (d) with ethanol (1:1, v/v). (*2*) *Oxidizing reagent:* Dilute an aliquot of 2,6-dichloroindophenol solution (d) with water (1:1, v/v). (This solution has an ascorbic acid equivalence of ca 88 μg/ml.)

(e) *Phosphomolybdic acid spray reagent.*—Obtainable from Brinkmann Instruments, Inc., Cantiaque Road, Westbury, N.Y. 11797.

(f) *Sodium acetate solution.*—Dissolve 25 g $NaOAc \cdot 3H_2O$ in water and dilute to 1 L.

(g) *o-Phenylenediamine solution.*—For each 100 ml of solution required, weigh 10 mg *o*-phenylenediamine dihydrochloride (No. 678, Eastman Organic Chemicals) and dilute to volume with water immediately before use.

Preparation of Sample Assay Solution

Perform *Preliminary Test for Appreciable Quantity of Basic Substances* and proceed as in *Preparation of Sample Assay Solution* described in previously published method (17), except in each case dilute sample assay solution to a concentration between 1.8 and 10 μg of each acid/20 μl.

Chromatography

Work in subdued light.

Lightly pencil a line 3 cm from bottom 7" edge of treated silicic acid-impregnated glass fiber paper (a). Apply standard and sample solutions on same paper with a 20 μl pipet, ca 4 cm apart. Dry spots by hanging paper in draft of hood for about 15 min. To avoid oxidation of the isomers, do *not* expose sheet to draft longer than necessary for drying.

Suspend paper in chromatographic tank with end, near which spots have been added, hanging free inside trough. Add ca 50 ml mobile solvent (b) to trough. Close tank with lid and develop until solvent front reaches top of paper (ca 25 min). Remove sheet from tank and dry in draft of hood just until solvent evaporates and immediately proceed under *Screening Qualitative Test* or *Determination.*

Screening Qualitative Test

Lightly spray sheet with phosphomolybdic acid spray reagent (e). Compare sample spots with standard spots applied to same sheet. Estimate sample concentrations by comparing sample spot size and intensity with that of the standards.

If sample contains only *l*-ascorbic or *d*-isoascorbic acid, re-chromatograph with respective standard (c)(*1*),(*2*) diluted to the estimated sample concentration. Proceed as in *Chromatography.*[3] If sample contains both

[3] Alternately, when only one isomer is present, the sample can be assayed without chromatography by the previously published method (17). If the isomer is *d*-isoascorbic acid, use standard solution prepared by substituting *d*-isoascorbic acid (Nutritional Biochemicals Corp.) for USP Reference Standard Ascorbic Acid, (b) in (17).

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l-ascorbic and *d*-isoascorbic acids, re-chromatograph with standard solution (c)(3) adjusted to the estimated sample concentration of both isomers. Proceed as in *Chromatography*.

Determination

Lightly spray sheet with chromogenic reagent (d)(1). Remove spots and blanks with ⅞" cork borer. Place in tubes 1⅛" i.d. containing 2 ml extracting solution ((e)(1) in (17)). Swirl gently. Add 0.25 ml oxidizing reagent (d)(2). Swirl and add 5 ml sodium acetate solution (f) with automatic pipetting machine. Remove disks and transfer contents to test tube containing 2 ml xylene. Shake to extract pink into xylene layer. Centrifuge. Remove xylene layer, swirl aqueous layer with Vortex mixer, and transfer 2 ml aqueous layer to each of three fluorescence reading tubes ((k) in (17)). Using automatic pipetting machine, add 5 ml *o*-phenylenediamine solution (g) to all tubes. Protect from light and let stand 35 min at room temperature.

Fluorometry

Compare samples containing only *l*-ascorbic acid or *d*-isoascorbic acid with respective standards (c)(1),(2) chromatographed individually. Compare samples containing both forms with standard mixture (c)(3) separated chromatographically.

Read fluorescence of standard and designate as A; next read fluorescence of sample and designate as B; then read fluorescence of blank and designate as C.

mg Isomer/sample unit = $[(B-C)/(A-C)]$ × dilution factor.

Results

Fruit beverages were assayed by the previously published method, using Norit oxidation and a concentration of about 10 μg/assay tube. The *l*-ascorbic acid found in the sample/20 μl is shown in the first column of Table 2. Twenty μl portions of sample with and without added *d*-isoascorbic acid were chromatographed and assayed by the modified procedure at concentrations of 0.4 to 2.3 μg/assay tube. Sample fluorescence readings were compared with analogous standards identically chromatographed and assayed.

Recoveries averaged 99% for *l*-ascorbic acid chromatographed alone and 102% from the mixture, compared to results obtained on the unchromatographed sample. The average recovery of *d*-isoascorbic acid was 104%. The greatest losses of isomers reportedly occurred during drying of the spot (13). *d*-Isoascorbic acid oxidizes more rapidly than ascorbic acid (2). Citric acid, which has shown strong ability to stabilize both forms (19), was added as an antioxidant to four samples, and stannous chloride was added to one sample. The higher recovery computed for *d*-isoascorbic acid may be the result of oxidation of the standard during drying of the spot, whereas the *d*-isoascorbic acid added to the sample was protected by the sample ingredients.

This paper was presented at the Eightieth Annual Meeting of the Association of Official Analytical Chemists, Oct. 10–13, 1966, at Washington, D.C.

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Table 2. Quantitative data pertaining to separation and assay of *l*-ascorbic and *d*-isoascorbic acids

Sample	*l*-Ascorbic Acid, μg/20 μl			*d*-Isoascorbic Acid, μg/20 μl	
		Chromatographed		Chromatographed	
	Unchromatographed[a]	Individually[b]	Sepd from Added *d*-Iso[c]	Added (By Wt)	Sepd from Sample Asc. Acid[c]
Grape drink, frozen concentrate	4.7	4.6	4.5	4.0	4.0
Pineapple-orange drink	3.0	3.1	3.0	4.0	4.2
Peach nectar	2.2	2.1	2.3	3.0	3.2
Pear nectar	2.4	2.6	2.5	2.0	2.0
Five-fruit blend	4.6	4.6	5.0	4.0	4.2
Cranberry juice	1.4	1.4	1.4	6.0	6.2
	2.7	2.6	2.8	8.0	8.0
Apple juice	5.2	5.1	5.2	1.8	1.9
	5.2	5.2	5.3	2.4	2.6

[a] Assayed as in Reference (17).
[b] Compared with *l*-ascorbic acid standard chromatographed individually.
[c] Compared with appropriate separated standard.

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Assay for Vitamin C: A Collaborative Study

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A collaborative study was conducted to evaluate the microfluorometric assay for vitamin C. Comparative assays for vitamin C were made, using a modified 2,6-dichloroindophenol assay and the 2,4-dinitrophenylhydrazine method. Results by the fluorometric method were good. Both the microfluorometric assay and the modified 39.040 method were recommended for adoption as official, first action.

Except for fruit and vegetable juices, there is no official assay for the analysis of reduced ascorbic acid in foods nor for the determination of total antiscorbutic activity in any product.

The simplicity of the official, final action method for ascorbic acid, 2,6-dichloroindophenol (DCP) titration, **39.040** (1), makes it practical for routine assays. As described, however, its use is limited. The method cannot be used for the determination of vitamin C in a variety of products, such as those containing an appreciable quantity of basic substances or a significant amount of dehydroascorbic acid.

The 2,4-dinitrophenylhydrazine (DNPH) method of Roe and Kuether (2) and the *o*-phenylenediamine (OPDA) method of Deutsch and Weeks (3) are based on the conversion of ascorbic acid to dehydroascorbic acid and direct measurement of total dehydroascorbic acid. The result obtained by either of these methods is the antiscorbutic activity of the sample.

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The Comparative Properties of Ascorbic Acid and Erythorbic Acid

FOTE 19-121

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SUMMARY

Although erythorbic acid has no vitamin C activity, it has been used interchangeably with ascorbic acid as an antioxidant in foods on the assumption that both compounds have equal antioxidant activity. The literature, however, demonstrates differences in chemical properties indicating that ascorbic acid is the superior antioxidant. Studies in model systems and in foods indicate that ascorbic acid is more stable in aqueous systems and is a more efficient antioxidant than erythorbic acid.

REVIEW

Ascorbic acid and erythorbic acid have frequently been used interchangeably as antioxidants in the food field on the assumption that the compounds have identical chemical properties. This paper examines this assumption by reviewing the basic and applied literature.

The literature on uses of ascorbic acid as a nutrient and antioxidant in the food field has been thoroughly reviewed by Bauernfeind (1953). The specific use of ascorbic acid as a flour improver has been reviewed by Tsen (1964). Erythorbic acid is ineffective as a flour improver because it cannot be used as a substrate by flour ascorbic acid oxidase or dehydroascorbic acid reductase. The biochemical properties and antiscorbutic activity of analogs of l-ascorbic acid have been discussed by Sebrell and Harris (1954). They reported that erythorbic acid has 5% of the antiscorbutic activity of l-ascorbic acid.

The function of ascorbic acid and erythorbic acid as antioxidants in food systems is: 1) to scavenge oxygen and thereby prevent oxidation of oxygen-sensitive food constituents; 2) to shift the redox potential of the system to a reducing range; and/or 3) to reduce undesirable oxidation products. The efficiency of ascorbic and erythorbic acids in food applications is determined by their redox potential, rate of oxidation, and stability during processing of the food.

Fig. 1 shows two-dimensional representations of four compounds: l-ascorbic acid, d-ascorbic acid, l-isoascorbic acid, and d-isoascorbic acid.

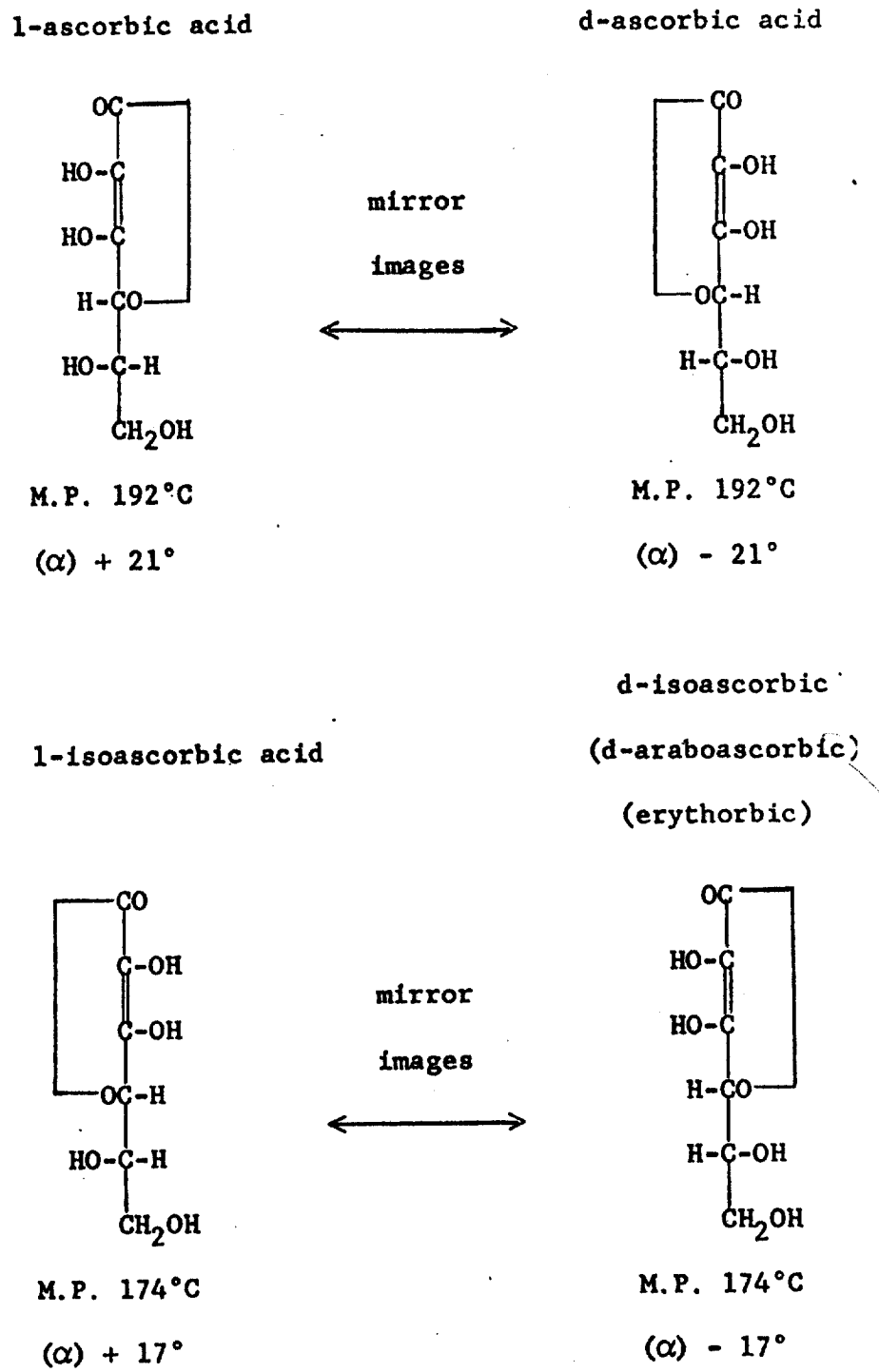

Fig. 1. The stereochemical configuration of ascorbic and isoascorbic acids.

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Table 1. Properties of l-ascorbic acid and erythorbic acid.

	l-Ascorbic acid	Erythorbic acid (d-iso-ascorbic acid)
Melting point	192°C	174°C
Solubility (g/100 ml of H_2O @ 25°C)	30	40
Solubility of sodium salt (g/100 ml of H_2O @ 25°C)	77	16
Redox potential $k^{\text{ox}}_{\text{red}}$ (pH 5.0 @ 25°C)	+.127v	*
oxidation	.166	.241

* Esselen (1945) reported that redox potential is different for the two compounds, but did not give absolute values.

The relationship among these compounds is confusing because of changes in nomenclature. The top two formulas are mirror images of each other. By definition, they have identical physical and chemical properties and differ only in their ability to rotate a plane of polarized light and in ability to partake in enzymatic reactions. There should be no difference in rate of oxidation, redox potential, melting point, solubility, etc. The two lower compounds are also mirror images of each other and have identical properties. The two products of commerce are l-ascorbic acid, with the melting point and magnitude of optical rotation shown in the upper left-hand formula, and d-isoascorbic acid, or erythorbic acid, which is the lower right-hand compound. These two compounds are not mirror images of each other. Their physical and chemical properties are summarized in Table 1.

Esselen *et al.* (1945) reported that erythorbic acid oxidizes more rapidly than l-ascorbic acid in food products and that the addition of erythorbic acid to tomato juice prevents the loss of the natural l-ascorbic acid content of the juice during storage. Similar results were obtained in a grape juice experiment in which both l-ascorbic and erythorbic acids were added prior to bottling—the erythorbic acid was preferentially oxidized. These experiments were suggested by Yourga *et al.* (1944), who found that erythorbic acid oxidizes more rapidly than l-ascorbic acid in buffered solutions.

Table 2. Schulte and Schillinger data, oxidation kinetics, Cu^{++} 2.8 ppm.

Buffer	pH	$k_1 \times 10^4$ (min^{-1}) 25°C d-Isoascorbic acid	l-Ascorbic acid
H_3PO_4+ Na_2HPO_4	3.0	240.0	120.0
	3.5	502.0	250.0
	4.0	1050.0	530.0
KH_2PO_4+ Na_2HPO_4	4.5	1740.0	* 955.0
	5.0	2410.0	1660.0
	5.5	3550.0	2560.0
	6.0	4200.0	3310.0
	6.5	3900.0	2880.0
	7.0	3110.0	2800.0
	7.5	2800.0	2860.0
	8.0	2900.0	2900.0

Table 3. Headspace oxygen and ascorbic acid content of apples canned with antioxidants at a rate equivalent to 300 mg ascorbic acid per pound (Hope data).

Antioxidant	Cortland Oxygen in headspace (vol. %)	Cortland Erythorbic and/or ascorbic acid (mg/100 g)	McIntosh Oxygen in headspace (vol. %)	McIntosh Erythorbic and/or ascorbic acid (mg/100 g)	Delicious Oxygen in headspace (vol. %)	Delicious Erythorbic and/or ascorbic acid (mg/100 g)
Ascorbic acid	0.33	23.48	0.40	18.83	0.63	24.55
Erythorbic acid	2.60	8.26	2.88	3.61	1.86	9.12
Sodium erythorbate	2.61	7.50	2.73	4.34	2.12	8.76
Check (no treatment)	12.05	1.75	10.84	0.75	11.34	2.75

Schulte and Schillinger (1952) reported a comprehensive study on the kinetics of oxidation of ascorbic acid and erythorbic acid in aqueous model systems in the pH range 3–9. They concluded that oxidation follows first-order kinetics in the pH range 3–7 in systems containing traces of copper, e.g., 1–7 ppm, and that erythorbic acid oxidizes 25–100% more rapidly than ascorbic acid under these conditions (see Table 2). They further concluded that their work substantiated the results of Esselen *et al.* (1945). As Table 2 indicates, both compounds are more stable in the pH range 3.0–4.5 than in the pH range 5.0–7.0. Stability can be improved markedly in model systems by reducing the copper content to parts per billion. In almost all food systems, however, oxidation of ascorbic acid and erythorbic acid can be considered to be copper catalyzed because of the presence of trace amounts of copper. Another effect of metals on these compounds is noted by Smith (1962), who found that 10% erythorbic acid solutions severely discolor stainless-steel no. 304 pipe, in contrast to 10% ascorbic acid solution, which causes only slight discoloration.

Hope (1961) evaluated the use of antioxidants in canning apple halves, the objective being to replace vacuum treatment with a more efficient oxygen-scavenging technique. He compared ascorbic acid, erythorbic acid, and sodium erythorbate at two concentrations. At an input equivalent to the oxygen content of the apples plus the can headspace, ascorbic acid treatment produced color and flavor superior to those with the erythorbates, but some discoloration was still noted. Although ascorbic acid was superior to erythorbic acid at this level, it was evident that oxidation of the reductants during processing necessitated using levels higher than stoichiometric. Hope therefore prepared products at a reductant level of 300 mg per pound of apples, with results shown in Table 3. These data were used to calculate oxygen retention in the canned product and reductant retention (see Table 4). As

Table 4. Reductant and oxygen stability in canned apples calculated from Hope data.

	Ascorbic acid	Erythorbate*
Residual oxygen in can (% of untreated control)	4%	25%
Reductant retained after processing (% of initial level)	34%	10%

* Mean of erythorbic acid and sodium erythorbate data.

corbic acid consumed 96% of the available oxygen; erythorbate consumed 75% of the available oxygen. Sixty-six percent of the ascorbic acid was oxidized in processing, versus 90% of the erythorbates. These experiments were carefully replicated.

Reyes and Luh (1962) compared the efficacy of ascorbic and erythorbic acids in retarding enzymatic browning of frozen peaches during 30 days, and found no differences in rate of browning in this period. They also studied the rate of degradation of the reductants and found that ascorbic acid is more stable than erythorbic acid in the product, confirming Schulte's work in model systems. Table 5 summarizes data on half life of the reductants at 34 and 68°F. The efficacy of reductants in enzymatic browning is due to their ability to reduce orthoquinone compounds back to the orthophenolic forms (Bauernfeind, 1958). When the l-ascorbic acid (or erythorbic acid) content is exhausted, the quinones polymerize, causing browning. The difference from Reyes and Luh's work is that erythorbic acid would be consumed 30 to 50% faster than ascorbic acid in peaches, and hence browning

would commence earlier in the erythorbate treatments.

The conclusions drawn from the above studies can be extended to other food applications where reductants are added to scavenge oxygen, react with an undesirable intermediate, or shift the redox potential. Pasteurized beer and vacuum-packaged cooked cured meats are analogous to canned apples in pH, processing conditions, and packaging. There are obvious chemical differences among the three systems, but in each case the primary reactant with reductants is oxygen.

Watts *et al.* (1955) demonstrated that added or naturally occurring reducing agents are essential to the shelf life of cured meat color, and that the concentration of natural reducing agents in cured meats varies greatly. The mechanism by which reducing agents are effective in stabilizing cured meat color is not definitively known. When reducing agents are added to meat prior to curing, part of the reducing agent will survive processing operations and retard degradative changes in the nitrosoheme pigments. Mills *et al.* (1958) studied the effect of sodium ascorbate and sodium erythorbate on the quality of frankfurters. Both compounds decreased processing time 32%. The authors did not determine residual reducing agents or color shelf life.

A limiting factor in the color shelf life of cured meats is the rate at which oxygen in the product or package reacts with the residual reducing agents and consumes them. When the reductant is depleted, color will fade rapidly, especially under light. The difference in oxidative stability of ascorbic versus erythorbic acid should favor a better color shelf life for cured meats processed with ascorbic acid or sodium ascorbate. Carefully controlled commercial tests substantiate this hypothesis. In all recent comparative tests run at our suggestion by technical personnel at commercial meat packers where analytical data have been obtained, residual ascorbate has been higher than residual erythorbate. In general, concentration of residual reductant has correlated with color shelf life.

The assumption that ascorbic and erythorbic acids have identical chemical properties is refuted by both the basic and applied literature. It would appear that ascorbic acid is a superior antioxidant in food applications, particularly in processes involving heat.

Table 5. Kinetic constants for loss of antioxidants during thawing of peaches (Reyes and Luh data).

Sample	Antioxidant	Antioxidant concentration in syrup (% by weight)	Velocity constant 34°F	Velocity constant 68°F	Half life (days) 34°F	Half life (days) 68°F
C	Ascorbic acid	0.15	0.63	1.84	110.0	38.0
F	Erythorbic acid	0.15	0.82	2.62	85.0	26.0

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NOTE 19–122

Discoloration of Canned Cured Meats Caused by Copper

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SUMMARY

Ox tongues were used in an investigation of a black discoloration caused by contamination of canned cured meats by copper. Traces of copper dissolved in curing brines became concentrated about 10-fold in the skin of the blade of ox tongues. Brines containing as little as 0.5 ppm copper produced noticeable discoloration of canned tongues; 5 ppm gave an intense black discoloration. Copper may be removed from brines by adsorption on finely minced meat or by stirring the brine over iron metal. Contaminated brines treated by these methods gave tongues of normal color. Much of the copper adsorbed on cured tongues may be removed by treating them with acid solutions before scalding and canning. Discoloration did not occur in contaminated tongues canned after the acid treatments, and organoleptic quality was not impaired.

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INTRODUCTION

Copper is frequently implicated in sporadic outbreaks of discoloration in canned foods. The incidence of discoloration induced by copper is related to the wide use of copper and copper alloys in the manufacture of

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THE RELEASE OF EPINEPHRINE FROM RABBIT ADRENAL MEDULLA CHROMAFFIN GRANULES

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THE release of catecholamines from isolated chromaffin granules has been shown to be influenced by a number of agents. In bovine adrenal chromaffin granules, various adrenolytic drugs,[1] and tyramine,[2] release catecholamines. In bovine splenic nerve granules, reserpine inhibited the catecholamine releasing action of tyramine and at low concentrations inhibited the spontaneous loss of epinephrine.[3] In the adrenal medullary granules of the cat and rabbit, reserpine had a moderate inhibitory effect on the spontaneous release of catecholamines.[3] The present experiments describe some of the properties of the release of epinephrine from the chromaffin granules of the rabbit adrenal.

Methods

For each preparation of adrenal medulla chromaffin granules the whole adrenal glands from a 2 kg New Zealand white rabbit, anesthetized with pentobarbital, were homogenized in a loose glass homogenizer with 30 ml of 0.3 M sucrose. The homogenate was then fractionated by a method based on that of Hillarp.[4] The small amount of tissue used, about 125 mg, precluded a rigorous separation of the chromaffin granules from mitochondria. Nuclei and debris were removed by centrifugation for 10 min. at 800 X g. Centrifugation for 10 min. at 10,000 X g gave a fraction containing the chromaffin granules. This sediment was gently rinsed twice with 10 ml of 0.3 M sucrose, fat adhering to the tube was wiped off. The sediment was then homogenized in 25 ml of 0.3 M sucrose. This suspension contained 1-2 μg of epinephrine and 1-2 μg of adeno-

sine triphosphate (ATP) per ml. One ml of the chromaffin granule suspension and 0.44 ml of 0.1 M potassium phosphate buffer pH 7.0 were incubated with the compounds being tested in a total volume of 2.2 ml of 0.3 M sucrose. The buffer and compounds were made up in 0.3 M sucrose. These mixtures were shaken for 1 hour at various temperatures in stoppered bottles in a water bath. After the incubation, a 1.5 ml aliquot was centrifuged 15 min. at 12,000 X g and washed twice with 2 ml of 0.3 M sucrose by centrifugation at 12,000 X g for 15 min. The preparation of the adrenal granule suspension and the washings after incubation were done at 0 to 5°. As determined by analysis of supernatants, neither the original granule suspension nor the washed granules after incubation contained any appreciable epinephrine not bound to particles. The sediments obtained after the last washing was suspended in 1 ml of 0.01 N HCl and epinephrine determined fluorometrically[5] using an Aminco Bowman spectrophotofluorometer with activation at 410 mμ and fluorescence at 530 mμ. The percent epinephrine remaining in the granules was calculated by comparison with a control kept at 0° at which temperature the granules are stable for several hours. ATP was determined by firefly luminescence[6] using firefly lantern extract purchased from Worthington Biochemical Corporation. All incubations and analyses were done in duplicate or triplicate. Ascorbic acid solutions were prepared just before use.

Results

The spontaneous release of epinephrine from the granules was more marked in saline than in sucrose. When 1 ml of the chromaffin granule suspension in 0.3 M sucrose was added to 1.2 ml of buffered 0.3 M sucrose and incubated for 1 hour at 20°, the epinephrine content of the granules decreased 22 percent. When 1.2 ml of buffered saline was substituted for the 1.2 ml of buffered 0.3 M sucrose, the epinephrine content decreased 79 percent (Table 1). The spontaneous release of epinephrine increased markedly with increasing temperature. The spontaneous release of epinephrine was inhibited by ethylenediamine tetraacetate (versene) and reserpine (Table 1). Euler and Lishajko[3] reported that

reserpine partially inhibited the spontaneous release of epinephrine from rabbit adrenal medullary granules in a 2-hour incubation at 20°. In our experiments, at 20° for 1 hour, reserpine almost completely prevented the loss of epinephrine.

TABLE 1

Conditions Effecting the Spontaneous Release of Epinephrine From Rabbit Adrenal Chromaffin Granules

Temperature	Additions	Percent decrease in epinephrine content of the granules
20°	none	22(27,20,20)
37°	none	95(95,96,94)
20°	10^{-4} M versene	3(0,0,9)
37°	10^{-4} M versene	50(52,48)
20°	10^{-5} M reserpine	9(4,14)
37°	10^{-5} M reserpine	53(42,49,54,44)
20°	buffered saline in place of buffered sucrose	79(79,79,79)

TABLE 2

Effect of Ascorbic Acid and Isoascorbic Acid on Rabbit Adrenal Chromaffin Granules at 0° and 20°

Molar Concentration	Percent decrease in epinephrine content of the granules		Percent decrease in ATP content of the granules
	0°	20°	20°
Ascorbic Acid			
0	0	25(27,24)	24(22,24,26)
10^{-6} M	4(0,8)	33(35,30)	
10^{-5} M	6(5,4)	65(61,69)	77(78,75)
2×10^{-5} M		95(93,93)	95(96,94)
10^{-4} M	61(63,66)	97(97,97)	
Isoascorbic acid			
10^{-5} M		81(74,84)	

Ascorbic acid and isoascorbic acid greatly increased the loss of epinephrine and ATP from the granules (Table 2). The depleting action of ascorbic acid at 20° was antagonized by versene, imidazole, ATP, and reserpine (Table 3). A number of other sugar acids, oxidizing and reducing agents, at a concentration of 10^{-3} M had no marked effect on the release of epinephrine from the chromaffin granules - gluconic acid, galacturonic acid, potassium ferricyanide, potassium ferrocyanide, sodium bisulfite, sodium sulfate, sodium thiosulfate.

TABLE 3

Effect of Various Compounds on the Release of Rabbit Chromaffin Granule Epinephrine and ATP by Ascorbic Acid at 20°

Molar concentration of additions	Percent decrease in epinephrine content of the granules	Percent decrease in ATP content of the granules
$2X10^{-5}$ M ascorbic acid alone	94(94,94)	94(94,94)
$+10^{-5}$ M reserpine	15(15,15)	27(28,26)
$+10^{-5}$ M reserpine	23(26,23,20)	28(23,30,30)
$+10^{-5}$ M reserpine	14(10,20,13)	
$+10^{-6}$ M reserpine	81(79,81,83)	
$+10^{-4}$ M versene	17.5(20,15)	20(22,18)
$+10^{-2}$ M imidazole	10(11,9)	7(5,9)
$+2.5X10^{-3}$ M ATP	18.5(18,19)	
$+4.5X10^{-4}$ M ATP	49(45,53)	

Discussion

No ready explanation can be given for the ability of ascorbic acid to release epinephrine from the chromaffin granules. However, this effect of ascorbic acid suggests that the oxidation reduction potential of the media is of importance in maintaining the integrity of the granule. The fact that, of the reducing agents tried, only ascorbic acid and isoascorbic acid caused release, indicates a degree of specificity of the reaction. The protective effect of versene on the chromaffin granules suggests that the system contains a metal promoting the release of epinephrine. Furthermore, of the compounds that an-

tagonized the effect of ascorbic acid - versene, imidazole, ATP, and reserpine, all but reserpine are known to have chelating properties suggesting that a metal may be involved in the action of ascorbic acid. The fact that versene and reserpine protect the granules against both spontaneous and ascorbic acid induced release of epinephrine, suggests that ascorbic acid may act by accelerating the same process involved in spontaneous release.

Reserpine would appear to have little in common with the other compounds antagonizing the effects of ascorbic acid. However, it is of interest to note that Kirshner[7] found both reserpine and versene inhibited the uptake of catecholamines by isolated bovine adrenal chromaffin granules. The experiments reported here serve as another illustration of the ability of reserpine to influence amine transport in chromaffin granules.

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