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Effect of Ascorbic Acid on Guinea Pig Skin Collagen Synthesis: II. Neutral and Acid Soluble Collagens, Mucopolysaccharides, and Amino Acid Composition

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Scorbutic guinea pigs incorporated approximately 15% as much ^{3}H-proline into total skin collagen as the positive controls and hydroxylated 66% as much of that incorporated. ^{35}S-sulfate incorporation into skin acid mucopolysaccharides was decreased 50% in scorbutic animals. Amino acid composition of collagens was similar to that of other vertebrates.

An effect of ascorbic acid on collagen hydroxyproline was localized in vitro by Robertson and Hiwett,[1] using normal and scorbutic guinea pig granuloma, and in vivo by Gould,[2] using sponge implants. Subsequently, ascorbic acid has been found to function in the in vitro hydroxylase reactions which hydroxylate proline[3-5] and lysine[6] in protocollagen. Thus the role of ascorbic acid has been broadly defined. The role of ascorbic acid in the maintenance of collagen, in the maturation or aggregation of the fibrils, and in the biosynthesis of acid mucopolysaccharides in the same mesenchymal tissue has long been implied but is still equivocal.[7-9] The dried appearance of the skin[10] of ascorbic acid-deficient guinea pigs was indicative of an extensive loss of water. Mucopolysaccharides hold approximately 20 times more water than does collagen, which suggested an impairment of mucopolysaccharide synthesis or function. Since a close relationship in the mesenchymal tissue exists between acid mucopolysaccharides and collagen, both these substances were studied simultaneously in guinea pig skin.

The uptake of ^{3}H-proline and conversion to ^{3}H-hydroxyproline in total extractable guinea pig skin collagen[10] was found to decrease with a decreased ingestion of ascorbic acid. This parameter has been studied in the earliest and intermediate forms of collagen,[11-13] that is, the collagens extracted with neutral salt and acidic buffers, respectively. The uptake of ^{35}S-sulfate into the total skin acid mucopolysaccharides was measured to determine any impairment in these closely intermixed connective tissue components under limited and optimal ascorbic acid intake.

The amino acid composition of the neutral salt, acid, and total extractable collagen of skin from normal and ascorbic acid deficient animals was investigated to determine the composition of collagen from guinea pig skin and to ascertain any short-term effect of ascorbic acid on the composition. The influence of ascorbic acid deficiency on noncollagenous protein synthesis was elucidated from the specific activity of serum and pancreatic proteins.

Materials and Methods

Male albino guinea pigs weighing approximately 250 gm were treated and fed as described previously.[10] In Experiment I, 16 days duration, two groups of four were used: one group (positive control) received the basal diet (Table 1) supplemented with 2,000 ppm ascorbic acid; the second group received ad libitum the diet containing no ascorbic acid but were given orally 0.5 mg ascorbic acid daily/100 gm body weight.

In Experiment II, 22 days duration, three groups of eight were used: one group received the basal diet (negative control), the second group received the basal diet supplemented with 2,000 ppm of ascorbic acid (positive control), and the third group

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TABLE 1

Composition of Basal Diet	gm/kg
Vitamin-free casein	300
Cornstarch	170
Sucrose	100
Solka Floc*	150
Cerelose†	111
Salt mixture‡	90
Corn oil	30
Vitamins A, D, E, K, in corn oil§	20
Water-soluble vitamin mixture in cerelose‖	20
Choline chloride	2
Inositol	2

* Powdered Cellulose obtained from Brown and Co. Berlin, NH.

† Glucose monohydrate obtained from Corn Products Co., New York, NY.

‡ This mixture had the following ingredients/kg. of salt mixture: 389 gm $CaPHO_4$, 67 gm $CaCO_3$, 64 gm NaCl, 87 gm KCl, 273 gm K acetate, 56 gm MgO, 51 gm $MgSO_4$ (anhydrous), 7.1 gm Ferric citrate, 4.2 gm $MnSO_4$ (anhydrous), 0.17 gm KIO_3, 0.056 gm $CuSO_4$ (anhydrous), 1.44 gm $ZnCO_3$. This furnished the following levels of minerals/kg of diet: 10.6 gm Ca, 6.7 gm P, 11.7 gm K, 3.3 gm Mg, 1.9 gm Na, 6.0 gm Cl, 100 μg Mn, 84 μg Fe, 1.7 μg Cu, 60 μg Zn, and 7.6 μg I.

§ The fat-soluble vitamin mixture furnished the following amounts/kg of diet: 6 μg vitamin A acetate (18,000 IU), 40 mcg vitamin D_3 (1,600 IU), 20 μg alpha tocopherol acetate, 2 μg 2-methyl-4, 6-naphthoquinone.

‖ The water-soluble vitamin mixture furnished the following amounts/kg of diet: 16 μg thiamine, 16 μg riboflavin, 16 μg pyridoxine, 40 μg calcium pantothenate, 200 μg niacin, 0.5 μg biotin, 10 μg folic acid, 50 mcg vitamin B_{12}.

received the positive control diet but was pair-fed with the negative control animals by giving them the same quantity of diet consumed the previous day by their negative control pair. The negative controls consumed the diet at 1 or 2 gm below the positive control group level.[10]

On the 15th and 19th day of Experiment I and II, respectively, the animals were injected intraperitoneally with a physiological saline solution containing both ^{3}H-proline (UL)* 0.75 μC/gm body weight and ^{35}S-sulfate†, 310 mC/mMole, 1 μC/gm body weight. The guinea pigs of Experiments I and II were exsanguinated on the 16th and 22d day, respectively. The skins were freed of hair, some by shaving prior to sacrifice with an electric clipper, quickly removed, cooled, put through an electric meat grinder twice, using ice to force the skins through, and were frozen at −20C. The technic of Piez, Eigner, and Lewis[14] was used for the extraction of the neutral salt-soluble collagen except that the tissue was extracted for two 48-hour periods at 4C with 25 ml of 1 *M* neutral sodium chloride/2 gm weight wet skin. The acid-soluble collagen was obtained from the residue of the salt extraction by shaking with approximately 10 volumes of 0.5 *M* sodium citrate buffer pH 3.5 for three days and re-extracting for another two days. The purification procedure of Piez et al[14] was followed for both extractions. The protein contents of the extracts were determined and 20 mg of each were hydrolyzed for separation of proline and hydroxyproline.[10] The technic for the analysis of protein, proline, hydroxyproline, and radioactivity was as previously described.[10]

For amino acid composition, equal quantities of lyophilized protein were oxidized with performic acid.[15] The solutions were dialyzed immediately in cold 0.15 *M* acetic acid overnight and lyophilized. After acid hydrolysis in constant boiling hydrochloric acid for 30 hours at 108C, the composition of amino acids was determined using an external standard of β-2-DL-thienylalanine and the usual procedure for the separation of amino acids.‡

Acrylamide gel electrophoresis[16] with three layers of gel was made in a commercial apparatus using sodium acetate buffer pH 4.5. All solutions were deaerated for 30 minutes prior to use, and a measured amount of gel solution was added to each 0.5 cm diameter tube by means of a long-tipped measuring pipette to give, lower to upper layer, 2 cm of 7.5% and 1 cm each of 5.5% and 4.5% gels. The gels polymerized in one to two hours under fluorescent lighting. The remainder of the procedure was as described by Veis and Anesey.[16]

The extraction of mucopolysaccharides from the dried defatted skin was according to the method of Schiller, Slover, and Dorfman,[17] except that sodium citrate buffer 0.04 *M* pH 6.8 was used for the papain digestion.[18] After the trichloroacetic acid precipitation, the total mucopolysaccharides were precipitated with 3 volumes of 95% ethanol and 5% potassium acetate at 4C overnight and were washed twice with ethanol, once with ethanol:ether, 1:1, and finally with ether, followed by drying in vacuo. Total uronic acids were estimated according to Bitter and Muir.[19] Hexosamines

* International Chemical and Nuclear Corp, City of Industry, Cal.

† New England Nuclear Corporation, Boston, Mass.

‡ AAA Laboratory, 14351 22d Avenue NE, Seattle, Wash.

were estimated in aliquots of mucopolysaccharides hydrolyzed for four hours at 95C in constant boiling hydrochloric acid. Total hexosamines were determined by the technics of Boas[20] and Gardell,[21] using a colorimetric benzidine technic.[22] Radiosulfur was determined on the hydrolyzate at an efficiency of 60%.

Serum obtained from blood collected at autopsy of the Experiment I animals was fractionated in three different ways to measure incorporation of ^{3}H-proline into the serum proteins: (1) Whole serum was well dialyzed in 0.14 *M* sodium chloride and protein and tritium were determined. (2) Approximately 50% of the whole serum protein was precipitated with 0.37 *M* trichloroacetic acid, washed once, redissolved in 0.14 *M* sodium chloride and the specific activity was determined. (3) Paper electrophoresis with veronal buffer pH 8.6 was used to separate the albumin and globulin, and quantitative protein estimation of the stained strips was made with a densitometer device, the bands were cut out, and radioactivity was determined with appropriate corrections for quenching.

The pancreases from all animals in Experiment II were rapidly excised and frozen at −20C. They were pooled, weighed, and minced at 0C then extracted with four volumes of cold 0.25 *N* (normal) sulfuric acid by shaking in ice for 15 minutes.[23] The supernatant solutions following centrifugation were adjusted to pH 5 and dialyzed overnight in a solution containing 0.005 *M* diisopropylfluorophosphate and 0.05 *M* sodium acetate pH 4.8. After centrifugation, the solution was analyzed for protein content at 280 mμ, using the extinction coefficient of $E^{1\%}_{280\,m\mu} = 18.0$ for pancreatic proteins.[24] ^{3}H-proline was assumed to account for the tritium present.

Results

The composite specific activities of ^{3}H-proline and ^{3}H-hydroxyproline incorporated into the neutral salt-soluble, acid-soluble, and total extractable collagens (Table 2) for animals maintained on the diets for 16 and 22 days indicated similar incorporation into the neutral salt-soluble collagen only, and only for the positive controls. Animals receiving less than optimum ascorbic acid levels (ie, 0.5 mg ascorbic acid, Experiment I, and pair-fed animals, Experiment II) incorporated 64% as much proline three days after injection or 22% after only one day, into the neutral salt-soluble collagen, with the same relative relationship holding for the hydroxyproline specific activity. Animals receiving no ascorbic acid for 22 days, three days, postinjection, had 15% and 17% specific activity of that of the positive controls for proline and hydroxyproline, respectively. The acid-soluble collagen and total trichloroacetic acid soluble collagens differed for the positive controls in that the one-day postinjection animals had approximately one half the specific activity of the three-day postinjection animals for both proline and hydroxyproline. The Experiment I animals receiving 0.5 mg ascorbic acid daily/100 gm body weight had approximately one half the specific activity of their corresponding controls. Three days postinjection, the negative control group had approximately 10% the specific activity of the positive control group for both the acid-soluble and total extractable collagen. However, their pair-fed controls exhibited es-

TABLE 2

SPECIFIC ACTIVITIES OF PROLINE AND HYDROXYPROLINE IN GUINEA PIG SKIN COLLAGENS

	Neutral Salt Soluble		Acid Soluble		Total Extractable	
	Disintegrations ^{3}H/Minute/μMole					
Treatment	Proline	Hydroxy-proline	Proline	Hydroxy-proline	Proline	Hydroxy-proline
1-Day Postinjection						
Positive control	4,020	1,729	761	480	874	695
0.5 μg ascorbic acid/100 gm	868	269	581	226	464	337
3-Day Postinjection						
Positive control	4,613	2,678	1,838	1,243	1,986	1,550
Negative control	695	459	167	115	324	168
Pair-fed control	2,936	1,439	1,821	1,249	1,601	1,238

sentially the same specific activity as did the positive controls.

An indication of the maturity of the collagen should be given by the percentage of specific activity of hydroxyproline to proline in the collagen. Table 3 shows that the progressive increase in the ratio of the specific activities occurred as the collagen increased in insolubility for all groups except the negative control animals. In the negative control group, the highest ratio was in the most soluble collagen and the least was in the insoluble collagen, indicating a decreased hydroxylation of proline, or possibly a block in intermolecular bonding required for insolubility.

The quantity of extractable skin collagen decreased for animals receiving no ascorbic acid, for their pair-fed controls, and for animals receiving 0.5 mg asorbic acid daily. However, if the percentage soluble to total collagen is calculated, the positive and negative controls showed 5% each for the neutral salt and 3.0% and 3.4% for the acid-soluble collagens. The pair-fed animals had 4.3% and 2.8% for the same extractions.

The total quantity of the soluble collagen extracted from the various groups of guinea pig skins (Table 4) showed a decrease for the animals receiving suboptimal ascorbic acid, either at the 0.5 mg dosage or none at all, but no decrease for the pair-fed group which was ingesting approximately 9 mg/100 gm body weight. The net synthesis of radioactive collagen as well as total collagen on a mg/gm of animal was decreased. The present results would suggest that ascorbic acid was necessary for the maintenance of neutral salt soluble and possibly for the other stages as well. The

TABLE 3

RATIO OF SPECIFIC ACTIVITY OF HYDROXYPROLINE TO PROLINE ×100 IN THREE COLLAGENS EXTRACTED FROM GUINEA PIG SKIN

Treatment	Neutral Salt Soluble	Acid Soluble	Total Extractable
1-Day Postinjection			
Positive control	43	63	80
0.5 μg ascorbic acid/100 gm	31	39	73
3-Day Postinjection			
Positive control	60	68	78
Negative control	71	69	52
Pair-fed control	49	69	77

TABLE 4

TOTAL HYDROXYPROLINE, RADIOACTIVITY FROM HYDROXYPROLINE AND COLLAGEN PROTEIN IN WHOLE GUINEA PIG SKINS

Treatment	Average Animal Weight gm	Average Wet Skin Weight gm	Neutral Salt Soluble			Acid Soluble			Total Collagen		
			Collagen μg	Hydroxyproline μMoles	DPM as HOP* ×10⁻⁴	Collagen μg	Hydroxyproline μMoles	DPM as HOP* ×10⁻⁴	Collagen μg	Hydroxyproline μMoles	DPM as HOP* ×10⁻⁴
1-Day Postinjection											
Positive control	284	14.3	196	200	34.55	77	79	3.80	2.86	2.93	205
0.5 μg ascorbic acid/100 gm	262	16.9	98	100	2.69	52	54	1.21	3.50	3.58	121
3-Day Postinjection											
Positive control	312	15.3	173	177	49.00	104	106	13.20	3.48	3.56	552
Negative control	203	10.1	60	61	2.80	42	43	0.50	1.24	1.27	21
Pair-fed control	260	13.2	110	113	16.20	61	62	7.74	2.54	2.59	321

* HOP as an indication for hydroxyproline.

22-day negative control animals had 35% to 40% of the total collagen of the positive control animals (Table 4) while consuming diet at 1 to 2 gm less per day. The pair-fed controls, while consuming the same quantity of ascorbic acid diet as did the negative controls,[10] had twice the amount of collagen, thus nullifying the factor of inanition. When calculated on a mg/gm animal-weight basis or on a mg/gm of wet-skin basis, the percentages are still in the same proportion (the calculations may be made from the data given in Table 4). However, the ratio of soluble to total collagen is essentially the same for all groups of animals. Current values for the 1 *M* neutral salt-soluble collagen mg/gm skin are approximately ten times higher than those calculated from the data of Bentley and Jackson[25] for their combined 0.14 *M* and 1.0 *M* salt extraction, but these values would be in relative agreement with the quantity found by Nimni[26] for the rat.

The analyses for the components of the skin acid mucopolysaccharides, uronic acid, hexosamines, sulfate, and radioactivity, on a dry-skin basis, are itemized in Table 5. The extremely high incorporation of ^{35}S after one-day postinjection will be discussed. In general, the specific activity of the ^{35}S-sulfate was approximately 50% of that of the positive control animals, for those receiving 0.5 mg ascorbic acid daily and for the negative controls, whereas the pair-fed group had the same specific activity as did the positive controls.

A decreased relative uptake of ^{35}S-sulfate in total skin acid mucopolysaccharides (Table 5) was noted for the animals receiving 0.5 mg ascorbic acid/100 gm body weight daily and for the negative controls. No quantitative change could be determined for the pair-fed animals subjected to the same inanition as the completely deficient animals, negative controls.

Amino acid analyses on a residue/1,000 residues basis are given for the three collagen extractions (Table 6). No differences in composition could be determined for the collagens from the negative and positive control groups; the figures given are the average of two determinations on performic acid oxidized samples. Ornithine was observed at 33 ml for total collagen; the other samples were not run in such a manner as to detect it. The purity of the collagens analyzed was confirmed by acrylamide gel electrophoresis; the neutral salt-soluble collagen had two extremely fine bands 1 mm apart which had migrated into the 7.5% gel 1 cm past the interface, and the acid-soluble collagen had one band at the interface between the 5.5% and 7.5% gels.

The uptake of ^{3}H-proline into the non-collagenous proteins of the serum (Experiment I) and of the pancreas (Experiment II) was determined. The results for the total dialyzed serum were as follows. The positive controls and 0.5 mg ascorbic acid animals: 2,820 and 2,780 DPM (disintegrations per minute)/mg protein; trichloroacetic acid precipitated proteins: 4,700 and 4,600 DPM/mg protein; and for incorporation into globulin: 3.2 and 4.9 in arbitrary units, respectively. The tritium activity of the pancreatic proteins for the three groups of Experiment II, as DPM/mg protein, was: positive controls, 4,525; negative controls, 6,935; and pair-fed controls, 6,665. Each figure represents the average from two pools of glands.

TABLE 5

ANALYSES OF GUINEA PIG SKIN ACID MUCOPOLYSACCHARIDES

Treatment	μ Moles/gm Dry Skin			DPM* as ^{35}S/gm Dry Skin	DPM*/μMole Sulfate
	Uronic Acids	Hexosamines	Sulfate		
1-Day Postinjection					
Positive control	3.87	3.71	2.41	231,000	96,000
0.5 μg ascorbic acid/100 gm	3.77	4.01	3.94	130,000	33,000
3-Day Postinjection					
Positive control	1.47	2.44	3.57	7,950	2,230
Negative control	0.72	1.87	2.39	3,350	1,400
Pair-fed control	2.21	3.06	3.42	7,750	2,270

* Disintegrations per minute.

TABLE 5

EFFECTS OF VARIOUS DIETARY LEVELS OF ASCORBIC ACID ON INDUCTION OF HEPATIC MICROSOMAL HYDROXYLATIVE ENZYMES IN GUINEA PIGS[a]

Treatment	No. of animals	Liver weight[b]	*O*-Demethylase[c]	Aniline oxidase[d]	Liver ascorbic acid[e]
2000 ppm Ascorbic acid	5	4.14 ± 0.26[f]	185 ± 12[f]	51 ± 6[f]	136 ± 21[f]
2000 ppm Ascorbic acid and 25 ppm dieldrin	4	4.59 ± 0.17[f,g]	414 ± 27[g]	92 ± 9[g]	110 ± 23[f]
50 ppm Ascorbic acid and 25 ppm dieldrin	4	4.96 ± 0.37[g]	240 ± 30[f]	49 ± 8[f]	27 ± 3[g]
200 ppm Ascorbic acid and 25 ppm dieldrin	5	4.68 ± 0.19[f,g]	353 ± 58[g]	75 ± 7[f,g]	33 ± 6[g]

[a] All animals were held 8 days on ascorbic acid-deficient diet then 4 days on the indicated treatments.
[b] Mean ± SE g liver weight/100 g body weight.
[c] Mean ± SE μmoles × 10^2 *p*-nitrophenol formed/g liver hr of incubation.
[d] Mean ± SE μmoles × 10^2 *p*-aminophenol formed/g liver 30 min of incubation.
[e] Mean ± SE μg ascorbic acid/g liver.
[f,g] Means for a particular variable which do not have any superscripts in common are significantly different at the 0.05 level.

DISCUSSION

There are numerous species differences in the inductive response to organochlorine pesticide ingestion. The responses observed in the present study were quite different from those seen previously in rats. DDT and lindane are rather potent inductive agents in rats (Schwabe and Wendling, 1967), but as shown here, are relatively weak in guinea pigs. Even the large proportional increases of *in vitro* enzyme activity following dieldrin consumption were not accompanied by consistent increases in liver weight or elevated microsomal protein concentration. Thus, the increased enzyme activity in guinea pigs may not be due to increased enzyme synthesis. Such species differences present difficulties to formulation of general principles and extrapolation of data from species to species, but they add to our knowledge of environmental effects of pesticides. They also provided more varied situations from which to select models of human responses.

It is concluded that ascorbic acid deficiency depressed induction of microsomal enzyme activity by organochlorine pesticides. However, for a number of individuals in experiments of 12–14 days duration the increment of enzyme activity produced by the inducer in animals fed vitamin-deficient diet (compared to vitamin-deficient controls) was nearly as great as that produced in those fed normal diets. Therefore, it is difficult to proportionally assign the consequences of ascorbic acid deficiency between effects on basal or on induced enzyme activities. A similar interpretive problem was reported by Marshal and McLean (1969) for the interaction of the inducing effects of phenobarbital and the depressing effects of protein deficiency. Presumably, a nutrient required to maintain normal enzyme activity would be required in greater quantity by the same enzyme system in a state of induction. Thus, deficiency of the nutrient should have a greater effect on induced enzyme activity than basal enzyme activity. However, it might

receiving any ascorbic acid, it would appear that collagen molecules are continuing to be hydroxylated after the formation of the 90,000 MW (molecular weight) subunits and into the aggregation of the insoluble stage. All the collagen fractions (Table 3) showed a gradual increase in the relative amount of radioactive hydroxyproline for all groups of animals except those which were completely deprived of ascorbic acid. The negative control animals, Experiment II, had the least uptake of radioactivity for all animals, but had a similar percentage conversion of proline to hydroxyproline in the neutral salt and acid-soluble collagens. However, in the total collagen, the negative control animals had only 52% conversion of proline to hydroxyproline of the possible 80% to 86%, inferring that the insoluble collagen was incompletely hydroxylated; but the amino acid composition for the collagens (Table 6) did not reflect a decreased proportion of hydroxyproline with respect to proline. Bornstein[33] has isolated incompletely hydroxylated peptides from rat skin and tendon collagen, although the amino acid composition of the entire α_1 chain of the same collagen did not differ significantly with respect to hydroxyproline. McLennan and Karasek[34] found increased amounts of total hydroxyproline to proline with increasing age in mouse skin.

There could be a feedback inhibition in scorbutic animals that would prevent synthesis of excess tropocollagen, thus not allowing an extremely high accumulation of the soluble collagen in these animals. Bentley and Jackson[25] have suggested that for 12-hour experiments, the collagen extracted with 1 *M* salt from scorbutic guinea pigs that had been on a scorbutigenic diet for 11 days does not show a decreased aggregation. In the present study, the recoveries of collagen from the whole skin also verifies the lack of any accumulation or enhanced decrease of one fraction over another.

Table 3 indicates that hydroxylation may be occurring after the 90,000 MW subunits are formed and even into the aggregation stage, which involves the size and conformational requirements of the peptide for hydroxylation. Nordwig and Pfab[35] have concluded that in the in vitro vertebrate systems no conformational requirements of the native molecule type need be considered for hydroxylation to occur, the only prerequisite being a specific sequence pattern. Lukens[36] found that the substrate for the cell-free hydroxylation reaction was comparable in size to the α or β components of ichthyocol, but he hastened to add that in vivo hydroxylation does not necessarily occur on chains of that length. Hydroxylation of the proline incorporated into the protocollagen chains occurs in vitro after a certain chain length has been attained, but this chain length is not yet clear.[3,30,37] On the other hand, it has been suggested that in vivo hydroxylation keeps pace with peptide synthesis.[38]

The decreased specific activity of hydroxyproline in the trichloroacetic acid extractable callagens from ascorbic acid deficient animals confirms previous reports with respect to ascorbic acid deficiency[2,30,39] and the synthesis of collagen in carrageenin-induced granulomas and sponge implants. In addition, this investigation showed a definite decrease in the synthesis of total collagen as defined by the incorporation of ^{3}H-proline not only into the trichloroacetic acid-soluble collagen but also into the earlier synthesized fractions; other laboratories reported no decrease in proline specific activity.[39] In the in vitro incorporation of proline into the microsomal collagen of normal and scorbutic guinea pig granuloma, Manning and Meister[30] have observed approximately the same reduction of incorporation for proline and hydroxyproline, 35% and 86%, respectively, that this report has observed in in vivo scorbutic guinea pig skin.

If the Experiment I groups are compared with each other and the Experiment II animals are compared with each other, no differences other than the radioactivity uptake can be seen. The reason for the different range of specific activity between the two experiments is not known. Schiller et al[10] calculated a half-life of approximately ten days for the rabbit and 10.7 days for the rat for chondroitin sulfuric acid with ^{35}S-sulfate. If the half-life of similar compounds were of the same order in the guinea pig, this would account for some but not all the difference observed.

The influence of ascorbic acid on the uptake of ^{35}S-sulfate in carrageenin-induced granulomas was studied by Kirchheiner,[41] who found for the scorbutic guinea pigs an increased uptake on day ten which de-

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creased to the control level by day 14. Kofoed and Robertson[42] determined the in vitro incorporation of ^{35}S-sulfate into sulfated glycosaminoglycans of tracheal cartilage and concluded that the caloric restriction in the presence of adequate ascorbic acid depressed sulfated glysaminoglycans synthesis to an even greater degree than that observed in advanced ascorbic acid deficiency. Such interpretations might be possible for the animals from Experiment I which received 0.5 mg ascorbic acid daily, or for the negative control animals of Experiment II which were imposing a slight inanition on themselves. However, the pair-fed animals in Experiment II did not show this deviation from the values for the positive controls, although they were being slightly starved at the same rate as were the negative controls but were receiving approximately 9 mg ascorbic acid daily/100 gm body weight.[10] Friberg[13] found that the incorporation of ^{35}S-label of chondroitin sulfate from scorbutic and pair-fed animals was about 28% that of normal animals and, for the recovery animals, about 43% of normal. In view of these divergent results, the answer must await the ultimate separation and purification of the individual mucopolysaccharide components. In the work reported here, it is of great interest that the uptake of ^{35}S-sulfate into the total skin mucopolysaccharides was similar in degree to the incorporation of ^{3}H-proline into the skin collagen. The difference between this work and that of others may be in the tissue studied or in the in vivo as compared to the in vitro synthesis.

The amino acid composition of guinea pig skin collagen (Table 6) is extremely similar to other vertebrate collagens.[14,44–47] The proteins were oxidized in order to determine the unequivocal presence of half-cystine residues. Calculated on a molecular weight basis of 90,000/α unit, there is one half-cystine residue on the average per unit. No differences were found in the composition of the collagens from the negative and positive control animals and the values were reported as duplicate analyses. Ornithine was reported by Steven and Jackson[46] in human achilles tendon collagen and in calf-skin tropocollagen; a comparable value of 2.5 was found in total collagen in the present study.

Since ascorbic acid does have an effect on collagen synthesis, its effect on noncollagenous protein synthesis was investigated by isolating serum and pancreatic proteins to determine any difference in the uptake of ^{3}H-proline. None was found for animals deficient for 16 and 22 days. Mitoma and Smith[48] investigated inductible enzyme formation in the liver and found that after 14 days scorbutic guinea pigs responded as well as the control animals. In view of the great deterioration of the animals on a scorbutic diet,[10] this lack of effect on noncollagenous proteins was somewhat surprising, but the proteins could be unique in their lack of response to ascorbic acid deficiency.

Conclusions

Guinea pigs fed a scorbutic diet for 22 days had a decreased uptake of ^{3}H-proline and ^{3}H-hydroxyproline into the neutral salt, acid, and total collagens as compared to animals receiving ascorbic acid. This decrease was approximately 85% for both amino acids (proline and hydroxyproline) for the negative controls and varied from 80% to 30% for those receiving 0.5 mg ascorbic acid daily/100 gm body weight. The decreased uptake for the pair-fed controls varied from approximately 40% to none.

Hydroxylation of ^{3}H-proline continued into the 90,000 MW stage and on into the insoluble collagen for all animals receiving any ascorbic acid. For the negative control animals, the hydroxylation was least for the insoluble collagen.

The total quantity of neutral, acid, and total collagen/animal was decreased during ascorbic acid deficiency, but the percentage of the total collagen was comparable for all groups.

The uptake of ^{35}S-sulfate into skin acid mucopolysaccharides decreased approximately 50% for animals receiving 0.5 mg ascorbic acid daily/100 gm body weight and for the negative control animals.

The amino acid composition of the neutral, acid, and trichloroacetic acid soluble collagens were extremely close to those from other vertebrates. Ornithine was found in the total collagen to the extent of 2.5 residues/1,000. One residue of cysteic acid/90,000 MW was found in all collagens.

The uptake of ^{3}H-proline into noncollagenous serum and pancreatic proteins was

not impaired by ascorbic acid deficiency.

The decreased incorporation of proline into protocollagen and the decrease of proline hydroxylation should be further investigated with respect to protocollagen synthesis and the protocollagen hydroxylase reaction. The apparent continuation of proline hydroxylation into the soluble stage would warrant further investigation of the ascorbic acid effect on intra- and intermolecular bonding.

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THE ASCORBIC ACID REQUIREMENTS OF SCHOOL-AGE GIRLS [1]

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ONE FIGURE

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During a recent investigation in this laboratory (Roberts and Roberts, '42) concerning the vitamin C requirements of children, five subjects were tested on intakes ranging from 55 to 135 mg. On the basis of blood and urinary excretion studies, it was found that 65 to 75 mg. of ascorbic acid was needed to insure saturation of these children. It seemed pertinent to extend the investigation to include a larger number of subjects in order to determine whether the majority of children in a larger group would respond in the same way.

It is well known that many children rarely get as much as 65 mg. of ascorbic acid in the diet. The subjects in this study were tested, therefore, on lower levels of intake than had been used in the earlier study.

EXPERIMENTAL

Selection of subjects. This investigation was carried on at a suburban home for girls. Thirty pre-adolescent girls between the ages of 6 and 12 years, living in two cottages, were selected as subjects. These children were in a healthy

[1] This research was aided by the Talcott fellowship granted by Rockford College and the Omicron Nu fellowship awarded by the American Home Economics Association to the senior author.

condition although, as in most groups of children, some were underweight, and some overweight. This deviation from average weight for height and age was taken into consideration, however, in the grouping of the children.

General procedure. The subjects were divided into six groups as based upon original ascorbic acid levels of the blood, age, height, weight, and deviation from average weight. The children from each group were given daily supplements of crystalline ascorbic acid [2] for three 7-day periods. The supplements ranged from 10 mg. for the first group to 60 mg. for the sixth group.

The amount of reduced ascorbic acid in the blood plasma was determined each week. At the end of the third period, the urinary "resting level" of ascorbic acid and the response to a 300-mg. test dose were measured. The blood concentration and the response to the test dose were the criteria used in judging the state of saturation of the subjects.

Control of the diet. Determination of the dietary intake for six of the subjects over a weekly period showed that the children were getting between 31 and 94 mg. of ascorbic acid with an average of 59 mg. On this intake their blood levels ranged from 0.46 to 0.97 mg. per 100 cc.

Because of the generous amount of vitamin C-rich foods served in the institution on some days, it was necessary to make a few dietary adjustments. Tomatoes, fresh and canned citrus fruits, all raw vegetables except carrots, and all fresh fruits except apples were eliminated from the diet. The menus were adjusted so that milk and dried fruit were the only sources of ascorbic acid for breakfast. Substitutions were made in the supper so that it, too, contained very little vitamin C. Most of the ascorbic acid was obtained at the noon meal where two or three vegetables and sometimes canned fruit were served. The children did not have access to food between meals.

[2] We wish to express our appreciation to Hoffmann-La Roche, Inc., Nutley, N. J., for furnishing the crystalline ascorbic acid.

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In order to determine the daily intake of ascorbic acid, the amount of food eaten by six of the subjects at the noon meal was weighed and samples of the food were analyzed. The milk and dried fruit from breakfast were also analyzed for vitamin C while the intake from the evening meal was estimated partly by analysis, and partly from tables of average composition. Inasmuch as the plates were served from the kitchen where the food had been divided quite evenly, and the children were required to "clean their plates," it seemed that the average obtained in this way would be fairly representative of the dietary intake of all subjects.

During the last 2 days of the study when urinary excretions were being determined, the diet was strictly controlled, and all foods were weighed and analyzed. In this way the intake of foods containing any vitamin C was kept the same for each subject. The average intake during each of the three weekly periods was 22, 21, and 22 mg.

Urinary determinations. The daily urinary output was collected for the last 2 days of the investigation, one day when the children were receiving their regular dietary supplement, and the other when they were given a 300-mg. test dose. To preserve the ascorbic acid in the urine, 5 N sulphuric acid and 8-hydroxyquinoline were used as suggested by Sendroy and Miller ('39).

Chemical methods. For the food and urine analyses, the method of Bessey and King ('33) was used. A buffer was added to the acidified urine to prevent the rapid fading of the end point. For blood determinations the microtechnique with the Farmer and Abt apparatus ('36) was used. All determinations were made on fasting blood plasma. The procedure of Menakér and Guerrant ('38) was employed for the standardization of the indicator, 2,6-dichlorophenolindophenol.

RESULTS AND DISCUSSION

In the present study, two criteria have been used for interpreting the results in terms of ascorbic acid requirements.

These are the same as were used in the more detailed study of Roberts and Roberts ('42). Blood levels of 0.7 mg. per 100 cc., and an excretion of 50% of a 300-mg. test dose within 24 hours are the two indices used to indicate a satisfactory state of nutrition in respect to vitamin C. Inasmuch as the daily excretion was obtained for only 1 day ascorbic acid "retention", which was one criterion in the earlier study, has not been considered in this report.

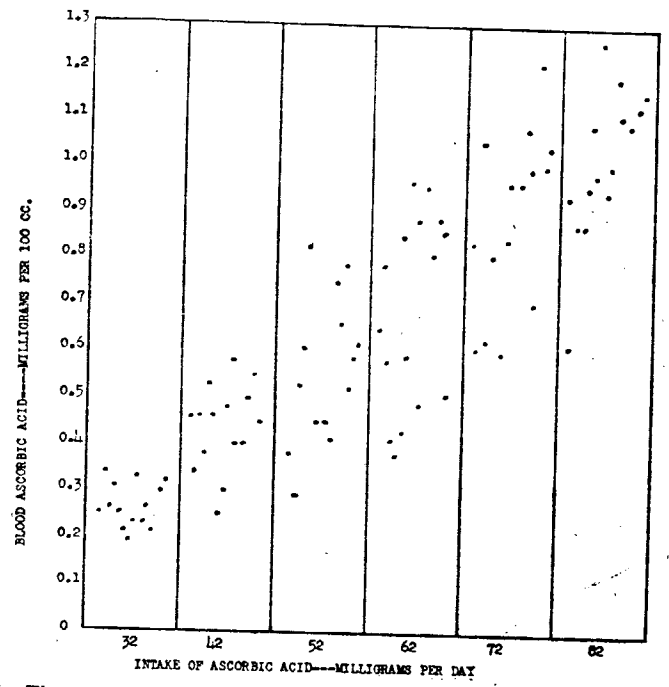

Fig. 1 The concentration of ascorbic acid in the blood plasma on various levels of intake. The figure shows results of three tests made at weekly intervals on each of thirty children.

A wide variation in blood ascorbic acid concentration on each level of intake is shown in figure 1. It will be noted that the blood levels are less variable on the lower than on the higher intakes. This variability has been observed by others. With boys living on an institution diet Roberts et al. ('39)

report blood levels ranging from 0.4 to 1.4 mg. per 100 cc. Bessey and White ('42) likewise found "considerable scattering" of blood concentrations at any one level of intake. No doubt these wide ranges are in part due to individual eating habits, and in part to variability in the response to vitamin C. Even with a controlled intake the individual blood

TABLE 1

Blood levels and excretion of ascorbic acid on various levels of intake.

GROUP	INTAKE		SUBJECT	BLOOD LEVELS MG. PER 100 CC.				EXCRETION	
	Average from food	Supplement		Periods 1	2	3	Average for 3 periods	"Resting level"	After 300 mg. test dose
	mg.	*mg.*						*mg.*	*%*
I	22	10	1	0.34	0.25	0.23	0.27	9	7
			2	0.25	0.21	0.26	0.24	7	15
			3	0.34	0.23	0.21	0.26	9	4
			4	0.26	0.19	0.30	0.25	8	22
			5	0.31	0.33	0.32	0.32	7	6
II	22	20	6	0.34	0.46	0.40	0.40	8	14
			7	0.46	0.25	0.40	0.37	10	22
			8	0.46	0.30	0.50	0.42	12	32
			9	0.38	0.48	0.55	0.47	14	39
			10	0.53	0.58	0.45	0.52	8	37
III	22	30	11	0.88	0.83	0.79	0.83	14	72
			12	0.38	0.45	0.52	0.45	10	77
			13	0.29	0.45	0.41	0.38	27	8
			14	0.53	0.75	0.59	0.62	13	90
			15	0.61	0.66	0.62	0.63	14	46
IV	22	40	16	0.65	0.85	0.96	0.82	23	78
			17	0.79	0.59	0.81	0.73	21	68
			18	0.58	0.97	0.89	0.81	28	65
			19	0.41	0.89	0.86	0.72	22	58
			20	0.38	0.49	0.51	0.46	10	32
V	22	50	21	0.84	0.60	1.05	0.83	31	71
			22	0.61	0.85	0.71	0.72	42	72
			23	1.06	0.97	1.23	1.09	34	79
			24	0.63	0.97	1.01	0.87	33	71
			25	0.81	1.09	1.05	0.98	14	75
VI	22	60	26	0.94	1.10	1.20	1.08	27	81
			27	0.62	0.94	1.12	0.89	..	..
			28	0.88	1.28	1.10	1.09	44	83
			29	0.88	0.95	1.14	0.99	22	45
			30	0.96	1.01	1.17	1.05	26	83

levels vary. With five subjects on the same diet Roberts and Roberts ('42) found differences as great as 0.3 mg. per 100 cc.

The blood levels and the urinary response of each child to a 300-mg. test dose are shown in table 1. Holmes, Cullen and Nelson ('41) have pointed out that repeated determinations of the blood ascorbic acid over a period of time constitute a more accurate measure of storage than a single determination. The blood levels during the three periods on the supplemented diet and also the mean value are included in the table. If a blood level of 0.7 mg. or above per 100 cc. is considered an indication of normal vitamin C nutrition, it is evident that the the children in groups I and II are subnormal. All of the children in these two groups were below 0.7 mg. per 100 cc. not only at the end of the first week, but during the entire 3 weeks of supplementation. Furthermore, the urinary "resting level" was low, and the response to the test dose was poor.

In the third group, only one child maintained a blood level above 0.7 mg. per 100 cc. Three in the group excreted 50% of the test dose. If the two criteria are applied as indications of vitamin C saturation, it is evident that subject 11 is the only child saturated on an intake of 52 mg.

The subjects in group IV who received 62 mg. showed a definite improvement. Though their blood levels were low during the first period, with the exception of subject 17, all blood levels increased during the second week, and these higher levels were maintained during the third period. Subject 20, however, is the only one whose mean blood level was below 0.7 mg. per 100 cc. Moreover, all except this girl excreted more than 50% of the test dose. No explanation can be offered as to the reason for this subject maintaining the low blood and excretion values. The urinary "resting level" for the other four subjects increased above the 20-mg. level, but fell short of the 40-mg. "resting level" considered by Van Eekelen and associates ('37) to be indicative of tissue saturation.

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That the next two groups, V and VI, were in a good state of vitamin C nutrition is evidenced by the high blood levels, the increased excretion, and the favorable response to the test dose. All had higher blood values at the end of the third period than they had at the end of the first, and their mean blood levels were above 0.7 mg. per 100 cc. With the exception of subject 29, all in these two groups excreted more than 50% of the test dose.

Examination of the mouths of the subjects by a dentist showed that there was no relation between either the intake or the blood levels of ascorbic acid and the condition of the gums. Three weeks on a low ascorbic acid intake is evidently too short a period to expect any visible tissue changes.

The data show that under the conditions set up, 52 mg. is the lowest intake of ascorbic acid on which any of the children were saturated: only one child, however, reached saturation on this intake. Four-fifths of the subjects receiving 62 mg., and all getting 72 mg., were saturated. Thus it appears that 62 to 72 mg. would be a satisfactory level at which to set the standards for the school-age child.

The results of this study are in fair agreement with other investigations on the school-age child. As judged by excretion studies, Harris ('40) found approximately 50 mg. of ascorbic acid sufficient to saturate boys 11 to 13 years old. Pemberton ('40) reports 63 mg. for boys from 10 to 14 years old. When judged by the response to the test dose alone, it will be noted in the present study that three of the five children receiving 52 mg. of ascorbic acid were saturated. Only one of these subjects, however, had a blood level above 0.7 mg. per 100 cc. Using the two criteria, 62 mg. was found more nearly adequate.

On the basis of blood studies Bessey and White ('42) report 40 to 50 mg. daily, sufficient to maintain a high plasma concentration. The intake was estimated, however, on the consumption of citrus fruit and tomatoes only. This amount could be better compared with the supplements used in the present investigation. On this basis there is close agreement between the results of the two studies.

Furthermore, this investigation substantiates the results found in the more detailed study of Roberts and Roberts ('42), where it was found that 65 to 75 mg. were required to saturate the subjects when the same criteria as were used in the investigation were employed to judge saturation.

Moreover, the values obtained here further corroborate the allowance set up by the Committee on Food and Nutrition of the National Research Council ('41).

SUMMARY

The ascorbic acid requirements of thirty pre-adolescent girls between the ages of 6 and 12 years were studied. Blood concentrations and the urinary response to a test dose were determined on intakes ranging from 32 to 82 mg. of ascorbic acid. Blood levels of 0.7 mg. per 100 cc. and the excretion of 50% of a 300-mg. test dose in 24 hours were used as the criteria indicating a satisfactory state of nutrition in respect to vitamin C.

None of the children on intakes of 32 or 42 mg. of ascorbic acid, and only one on an intake of 52 mg. were receiving sufficient vitamin C to maintain these standards. Four-fifths of those receiving 62 mg. and all who received 72 mg. were saturated.

On the basis of this study 62 to 72 mg. of ascorbic acid would seem to be an adequate allowance for pre-adolescent girls of 6 to 12 years.

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THE INDOPHENOL-XYLENE EXTRACTION METHOD FOR ASCORBIC ACID AND MODIFICATIONS FOR INTERFERING SUBSTANCES*

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There is no lack of methods for the determination of ascorbic acid, but none gives any great assurance of specificity. The indophenol reduction methods are probably the simplest, and these have been checked with animal assays for a number of fresh and cooked vegetables. The most generally adopted indophenol method has been that of Bessey (1), a method in which the excess dye is measured by photoelectric means, thereby offering a greater degree of objectivity than with the titration procedure. Provisions are made for correction of color and turbidity in the tissue filtrates and for substances which reduce the dye more slowly than ascorbic acid. With certain products, particularly fruits, the color of the blank may be several times that of the dye, leading to inaccuracies in the color measurements. From a practical standpoint, the need of two photoelectric measurements for each determination, and the time and care required for immediate readings and observation of possible "drifts" due to other reducing substances, are excessive when large numbers of determinations must be made. Some of these disadvantages have been eliminated by use of the principle described by Bukatsch (2) who noted that the excess oxidized dye may be extracted with xylene, and methods employing this principle have been described by Stotz (3) and Pepkowitz (4).

None of these methods, however, offers the desired specificity in the presence of a variety of other reducing substances which may occur in various heated, stored, and processed foods. Such non-ascorbic acid reducing substances include stannous and ferrous salts, sulfite, sulfhydryl compounds, reductic acid, and the reductones. Some of these, particularly the reductones, are most difficult to distinguish from ascorbic acid. Levy (5) has proposed the use of peroxide to eliminate sulfite interference. To account for reductones, Mapson (6) and Snow and Zilva (7) have devised methods which in principle involve condensation of the ascorbic acid, but not of the reductones, with formaldehyde. The use of the dinitrophenylhydrazine procedure of Roe and Kuether (8) and Roe and Oesterling (9), although eliminating some of the reducing materials, introduces other types of interfering substances.

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In this paper, an improved indophenol-xylene extraction method is described, and peroxide and formaldehyde modifications are presented which minimize the interference due to reduced tin and iron, sulfite, and reductones. This increase in specificity has been made possible without unduly complicating the routine usefulness of the method. Applications of the method to selected food products are given, and the extent of interference by thiol and quinol compounds is recorded. The basic method described and its modifications have been found satisfactory and convenient in this laboratory for the past year in the routine analysis of foods, particularly highly colored products, and those containing significant proportions of non-ascorbic acid reducing materials. We have noted sufficient instances of food products containing large proportions of non-ascorbic acid reducing substances so that we now consider it essential to apply regularly the procedures described, especially with food products which have been subjected to long heat treatments or extensive storage, before accepting values obtained by an unmodified indophenol method.

Procedure

Reagents—

Acetate buffer, pH 4.0. Mix 1 liter of 50 per cent sodium acetate ($NaAc \cdot 3H_2O$) and 1 liter of glacial acetic acid.

Dye. Dissolve 25 mg. of 2,6-dichlorobenzenone indophenol in 200 ml. of warm distilled water, and filter. The solution keeps in the refrigerator for at least 2 weeks.

Xylene. Unless specifically tested for suitability, this should be redistilled (see "Testing of method"). (Eastman Kodak technical grade has been found satisfactory if distilled.) Xylene used in the method may be recovered by being shaken with a 20 per cent solution of sodium hydroxide to neutralize acetic acid, and redistillation.

Peroxide, 3 per cent. Dilute 30 per cent C.P. hydrogen peroxide 10-fold. Prepare weekly.

Formaldehyde, technical grade, 40 per cent solution.

Hydroquinone. Add an equal volume of acetone to a saturated solution of hydroquinone in acetone, to obtain a half saturated solution. This must be prepared fresh before use.

The specific methods described apply to extracts of tissues prepared with fresh 2 per cent metaphosphoric acid in 1 N sulfuric acid. When other types of acid extractions are employed, the analyst should adjust the dilution of the acetate buffer to obtain a final pH of 3.6 to 3.8 in the basic method and its modifications.

With each new product to be analyzed, it should be determined whether the basic method (Procedure A) may be employed, either without or with a hydroquinone blank, or whether the peroxide modification (Procedure

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B) or formaldehyde modification (Procedure C) should be used. To determine whether the acid filtrate contains pigments which are extracted by xylene, the basic method (Procedure A) is applied to the filtrate without the addition of dye, and the xylene extract examined for color. Filtration of the extract through a fairly retentive paper (Whatman No. 2) usually removes the finely suspended particles containing carotene which occur with some products, and which otherwise result in slightly yellow xylene extracts. We have thus far encountered xylene-soluble pigments only in the filtrates of certain food products subjected to long heating, which then required the hydroquinone blank described under Procedure A. In such cases the quantity of pigment extracted depended on the time of shaking; hence the hydroquinone decoloration procedure was considered more accurate than to employ a blank without the dye, as suggested by Pepkowitz (4).

If the product is known to contain sulfite as a preservative, or to contain iron or tin from long storage in cans, the peroxide modification (Procedure B) may be routinely used with little extra trouble. Possible interference by tin or iron can be detected by a comparison of the results of applying Procedures A and B on the same sample. A similar comparison of results with the basic method (Procedure A) and the formaldehyde modification (Procedure C) will determine whether reductones are present in appreciable amounts. The latter may occur commonly in various processed and stored food products (see Table II), but much less frequently in fresh products.

Proof of the applicability and limitations of these methods, with mixtures of ascorbic acid and the various interfering materials, is given under "Testing of method."

Basic Method, Procedure A—A 1 to 5 ml. sample of acid filtrate, containing 0.02 to 0.15 mg. of ascorbic acid, is placed in a 150 × 18 mm. test-tube. An equal volume of acetate buffer is added (this is sufficient to adjust 6 per cent HPO_3 or 2 per cent HPO_3 in 1 N H_2SO_4 to pH 3.6 to 3.8), followed immediately by 2 ml. of dye and complete mixing; 10 ml. of xylene are at once added, a cork is inserted, and the tube is shaken vigorously for 6 to 10 seconds to extract the excess dye. A second tube ("total dye") to determine the amount of dye added is prepared by the same treatment of a volume of extracting medium equivalent to the sample employed in the determination. A standard tube is likewise prepared which contains 0.100 mg. of ascorbic acid in the same volume of extracting medium. Upon completion of a series, the tubes are centrifuged briefly to separate the layers sharply, and the top xylene layers are poured off or withdrawn and measured in a suitable photoelectric instrument at 500 mμ or with a filter which transmits maximally at this wave-length. Xylene solvent is used as a reference sample.

Since ascorbic acid may be quite unstable after addition of buffer of

pH 4.0, and excessively long contact with the dye permits the reaction of slower reducing substances to become appreciable, it is essential to add buffer, dye, and xylene in rapid succession. The time required for complete interaction of ascorbic acid and dye elapses during the mixing of reactants and addition of xylene. In a series of determinations, the operation upon each tube is therefore completed through the xylene stage before proceeding with the successive sample of acid filtrate.

If preliminary tests show that xylene removes color from the acetate-buffered tissue extract, then an additional operation is required with the sample tube. After the first reading, 2 drops of hydroquinone (in acetone) are added to the xylene extract and mixed. The orange color due to the excess dye is bleached within 30 seconds, after which a second reading is taken to measure the blank due to the tissue extract. If the xylene layers are transferred to a single cuvette for the photoelectric measurements, it is advisable to read a complete series, then to return the xylene layers to the original tubes, and to add hydroquinone to the whole series before reading the tissue blanks. This eliminates the possibility of hydroquinone from one sample contaminating a successive one containing dye in the xylene layer.

Peroxide Modification, Procedure B—To eliminate the reducing action of sulfite or reduced iron and tin, peroxide is added prior to the dye. For this purpose, a volume of 3 per cent hydrogen peroxide equal to the acetate-buffered extract, or a smaller volume of more concentrated peroxide sufficient to result in a 1.5 per cent solution, is added just before the dye. The remainder of the determination follows as in the basic method. Total dye and standard tubes are prepared as previously described, containing a total volume before dye addition equivalent to that of the sample tube.

Formaldehyde Modification, Procedure C—To correct for the action of reductones, the reducing action of ascorbic acid is selectively abolished by interaction with formaldehyde at pH 3.6 to 3.8 for 10 minutes. In practice, two tubes are required for the determination. The first, Tube 1, contains the selected volume of tissue filtrate; the second, Tube 2, containing the same volume of filtrate, receives an equal volume of acetate buffer, followed by a half volume of 40 per cent formaldehyde (*e.g.* 2 ml. of sample, 2 ml. of buffer, 1 ml. of formaldehyde) to give an 8 per cent formaldehyde solution. The tubes are allowed to stand at room temperature for 10 minutes. At the end of this period, Tube 1 receives its volume of acetate buffer and a half volume of *water* to yield a volume equivalent to that of Tube 2. 2 ml. of dye are then added to each tube, mixed, and the excess dye extracted with 10 ml. of xylene. The difference between the amounts of dye reduced in the two tubes represents the true ascorbic acid.

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Neither peroxide nor formaldehyde affects the stability of the dye in the xylene layer.

Calculations

Dye Equivalent—The amount of dye equivalent to a standard amount of ascorbic acid must be determined for the particular instrument and colorimeter tubes employed, and should be checked frequently. Amounts of dye present in the xylene extract are conveniently expressed as the log I_0/I (absorption) at 500 mμ. Should the instrument invariably be set at 100 (I_0) with xylene solvent, then log I_0/I may also be expressed as 2 minus log I, where I is the per cent transmission of the sample tube. The dye equivalent of ascorbic acid may be calculated from the total dye and standard tubes as follows:

$$0.1 \text{ mg. ascorbic acid} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{standard}}$$

$$0.1 \text{ mg. ascorbic acid} = \Delta \log_{\text{standard}}$$

$\Delta \log_{\text{standard}}$ ranges from 0.290 to 0.330 with the instruments employed in this laboratory.

For Basic Method and Peroxide Modification—The dye used by the ascorbic acid in the sample is

(1) $$\Delta \log_{\text{sample}} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{sample}}$$

The amount of ascorbic acid in the sample tube is then

(2) $$0.1 \text{ mg.} \times \frac{\Delta \log_{\text{sample}}}{\Delta \log_{\text{standard}}}$$

Provided I_0 is always 100, a simple graph may be utilized by plotting per cent transmission (on a logarithmic scale) against mg. of ascorbic acid (on a linear scale). A straight line is drawn between the points 0 ascorbic acid-per cent transmission of the total dye tube and 0.1 mg. of ascorbic acid-per cent transmission of the standard tube. Transmissions of sample tubes may then be converted to ascorbic acid values directly from the graph.

If hydroquinone is employed in the basic method, then log I_0/I_{sample} in Equation 1 must be replaced by

$$\log I_0/I_{\text{sample}} - \log I_0/I_{\text{hydroquinone bleached}}$$

For Formaldehyde Modification—

(3) $$\Delta \log_{\text{total reducing substances}} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{Tube 1}}$$

(4) $$\Delta \log_{\text{reductones}} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{Tube 2}}$$

(5) $$\Delta \log_{\text{ascorbic acid}} = \log I_0/I_{\text{Tube 2}} - \log I_0/I_{\text{Tube 1}}$$

From each Δ log value, the mg. of material (expressed as ascorbic acid) can be calculated by use of Equation 2.

Testing of Method

Basic Method—The principle of the basic method, namely the complete extraction of the oxidized dye by xylene, has been adequately tested by Bukatsch (2), Stotz (3), and Pepkowitz (4). Nelson and Somers[1] noted that the color of the xylene layer may increase on standing (dye oxidation) with some samples of solvent. It is therefore important either to test the suitability of the sample of xylene employed, or to redistil routinely the xylene as recommended in this paper. The finding of a linear relation between the log of absorption of the dye in xylene and the amount of ascorbic acid has been repeatedly confirmed in this laboratory. The use of the basic method permits the analysis of extracts of such highly colored food materials as beet, cherry, raspberry, and grape, without resort to expensive and time-consuming electrometric methods.

Peroxide Modification—Sulfite is able to reduce the dye under the conditions of the test, and this may occur in sulfite-treated foods in amounts which affect the accuracy of the method. In the peroxide modification of the titrimetric procedure employed by Levy (5), an error may arise owing to oxidation of the reduced dye by the peroxide. The end-point may vary according to the speed of the titration. The peroxide technique is particularly adapted to the xylene method, owing to the short contact of the dye with peroxide.

Reduced tin or iron also acts like ascorbic acid in dye reduction methods, and may occur in canned products, particularly acid products stored for considerable periods. Some of the increases in ascorbic acid noted on storage in cans may actually arise from the contamination with iron or tin.

Table I illustrates the interference caused by iron, tin, and sulfite, and the correction effected by the simple peroxide modification.

Formaldehyde Modification—Some fresh products, but particularly products which have undergone extensive heat treatment or long storage, may contain substances collectively termed reductones. It has been difficult to distinguish these from ascorbic acid, even by enzymatic means. Although these substances have not been identified chemically, it seems probable that they resemble the glucose-amine type of compound rather than the reductones produced by the action of alkali on sugars or the reductic acid formed in the reaction of pentoses and strong acid (10).

Mapson (6) has observed that formaldehyde condenses with ascorbic acid to form a complex that no longer reduces the indophenol dye. Snow

[1] Nelson, W. L., and Somers, G. F., personal communication.

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and Zilva (7) have made a careful study of the rate of formaldehyde condensation with varying concentrations. Their experiments showed that at pH 3.5, with an 8 per cent formaldehyde concentration, the ascorbic acid was almost entirely combined, while reductones and reductic acid were only 15 and 5 per cent combined, respectively. These methods involve procedures not readily amenable to routine analysis. Our study of the formaldehyde reaction with regard to time and pH led to the simple adaptation recommended in this paper.

As shown in Table II, a significant proportion of the reductones, reductic acid, and glucose-amine complex, artificially prepared, still acts like ascorbic acid in the formaldehyde modification. On the other hand, the naturally occurring reductones of stored dehydrated cabbage appear to be more sharply distinguished from ascorbic acid in their relation with formaldehyde,

TABLE I

Interference Caused by Reduced Iron, Tin, and Sulfite, and Its Correction by Peroxide (Procedure B)

0.10 mg. of ascorbic acid in each tube.

Interfering material	Molar ratio of material to ascorbic acid	Procedure A, "ascorbic acid"	Procedure B, ascorbic acid
		mg.	*mg.*
None		0.099	0.100
Fe^{++}	1:1	0.138	0.099
	1:1	0.121	0.101
Sn^{++}	1:1	0.104	0.100
	20:1	0.117	0.098
$SO_3^{=}$	10:1	0.114	0.100
	25:1	0.134	0.098
	100:1	0.205	0.099

one sample of which showed no combination with formaldehyde. Until the exact nature of the non-ascorbic acid reducing materials is recognized in different products, it cannot be claimed that the formaldehyde modification described offers an exact analytical discrimination between these materials and ascorbic acid. Nevertheless the procedure provides a closer approximation of the true vitamin value, and has provided us with a much greater assurance that our analyses represent true ascorbic acid.

Table II also illustrates the great discrepancies that may result from the conventional dye reduction analysis of several food products, and the more nearly correct values obtained by the formaldehyde modification. It is advised that food products be first tested by the formaldehyde modification to determine the possible presence of significant amounts of non-ascorbic acid reducing materials before adopting a simple dye titration or the basic method of this paper.

Other Interfering Substances—There remain other substances which may be encountered in the analysis of foods for which no adequate corrections are available, or for which specialized methods are necessary. Cysteine and reduced glutathione, at an equimolar concentration with ascorbic acid, increase the ascorbic acid result by 7 to 9 per cent, and at a 10:1 molar concentration by 40 to 50 per cent. These sulfhydryl compounds, like ascorbic acid, condense with formaldehyde at pH 3.6 to 3.8, but, if known to be present in interfering concentrations, can be eliminated by formaldehyde condensation at pH 0.6 (6). At the latter pH, these compounds

TABLE II

Interference Caused by Reductones and Related Compounds, Correction by Formaldehyde (Procedure C), and Illustrative Applications

Material	Apparent ascorbic acid (no formaldehyde)	Ascorbic acid (with formaldehyde modification)
	mg. per 100 gm.	*mg. per 100 gm.*
Ascorbic acid	5.0	5.0
Synthetic reductone solution (11)*	24.0	8.4
Reductic acid solution (12)	1.9	0.30
Glucosamine complex†	3.2	0.35
Dehydrated cabbage, light color	139	131
" " dark "	71	0
" " " "	60	4.2
Blackberry jelly	6.2	1.8
Guava-mix "	20.6	14.6
Apple-raspberry juice, 0° storage	1.1	0
" " 37° "	2.8	0.17
Apple juice	1.1	0.18
Grapefruit juice	28.1	28.0
Sauerkraut "	16.4	13.5

* The figures in parentheses represent bibliographic reference numbers.

† Prepared by warm storage of a solution containing glucose, glycine, phosphate, and malic acid.

condense with formaldehyde; ascorbic acid does not. Thiourea, addition of which has been suggested to prevent darkening of fruits (13), does not interfere at a molar ratio of 10:1.

In the commonly used hydrogen sulfide reduction method for dehydroascorbic acid, it is known that the quinols and sulfhydryl compounds formed may interfere with the dye reduction method. As quinol types we have tested catechol, tannic acid, pyrogallol, and hydroquinone. Catechol, at a 100:1 molar ratio with ascorbic acid, and tannic acid, at 10:1, cause insignificant errors; pyrogallol and hydroquinone, at 10:1, cause 25 to 50 per cent increases in the ascorbic acid values. Unfortunately their

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rate of reaction with the dye depends on the amount of ascorbic acid present. No suitable means have been found to correct for the error due to these substances.

SUMMARY

1. A rapid, accurate indophenol-xylene extraction method for the determination of ascorbic acid in common and highly colored food products has been described.

2. Interference by artificially prepared reductone and reductic acid has been largely eliminated. Normally occurring non-ascorbic acid reducing material, at least that in dehydrated cabbage, appeared to be completely differentiated from ascorbic acid by formaldehyde.

3. The extent of interference from sulfhydryl and quinol compounds is discussed.

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THE DETERMINATION OF DEHYDROASCORBIC ACID AND ASCORBIC ACID IN PLANT TISSUES BY THE 2,4-DINITROPHENYLHYDRAZINE METHOD

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In the customary method for the determination of dehydroascorbic acid the amount of ascorbic acid is estimated by oxidation-reduction techniques before and after reduction with H_2S. King (1) has shown that certain ketones and aldehydes on treatment with H_2S form compounds that respond like ascorbic acid in oxidation-reduction procedures, leading to a considerable error.

In a method, developed in this laboratory, for the determination of total vitamin C (2) an acid extract of tissue is passed through norit; this oxidizes ascorbic acid to dehydroascorbic acid, the bis-2,4-dinitrophenylhydrazone of which is treated with sulfuric acid, and the resulting red color is measured photometrically. We have adapted these analytical principles to the determination of dehydroascorbic acid in the presence of ascorbic acid. The procedure is similar to the above except that the acid-extracting solution contains 1 per cent thiourea and the oxidation by norit is omitted. The purpose of the thiourea is to stabilize the ascorbic acid during the extraction and subsequent treatment. Pure ascorbic acid dissolved in a solution containing 1 per cent thiourea and 5 per cent metaphosphoric acid, in a concentration of 15 γ per cc., gives no color after 5 hours coupling treatment with 2,4-dinitrophenylhydrazine at 37°. The assumption that no dehydroascorbic acid is formed from the ascorbic acid in plant tissues under the specified conditions is supported by the finding of some very low values for dehydroascorbic acid (Table I).

EXPERIMENTAL

Plant tissues purchased in a city market were ground in 5 per cent metaphosphoric acid solution. The ascorbic acid was photometrically determined in this extract by means of indophenol buffered at pH 3.6, according to the suggestion of Bessey (3). To 1 cc. of metaphosphoric acid filtrate in a colorimeter tube in an Evelyn colorimeter were added 9 cc. of indophenol containing appropriate amounts of sodium acetate according to the technique of Evelyn, Malloy, and Rosen (4). This procedure gave the values shown in Column 1 of Table I. Another portion of the filtrate was

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TABLE I

Comparative Analyses of Plant Tissues for Vitamin C

The values are expressed as mg. per 100 gm. or 100 cc. of material.

Tissue	Ascorbic acid	Ascorbic acid + dehydroascorbic acid	Dehydroascorbic acid	
	Indophenol photometric method (1)	2,4-Dinitrophenylhydrazine method (2)	By difference (2) − (1) (3)	Direct 2,4-dinitrophenylhydrazine (4)
Apple	3.1	4.5	1.4	0.0
Rhubarb	1.6	5.0	3.4	0.4
Lemon juice	57.6	59.5	1.9	0.5
Potato	28.1	31.6	3.5	0.5
Turnip	27.2	30.0	2.8	0.7
Asparagus	19.7	20.8	1.1	1.0
Carrot	5.4	8.3	2.9	1.0
Peach	1.3	3.3	2.0	1.0
Plum	1.2	3.6	2.4	1.2
Pineapple	15.6	21.0	5.4	1.3
Green peas	17.5	19.6	2.1	1.3
Cucumber	2.2	5.5	3.3	1.4
Grapefruit juice	40.3	45.0	4.7	1.6
Spinach	54.0	54.0	0.0	1.8
String beans	16.0	16.4	0.4	1.9
Lima beans	39.6	43.5	3.9	2.0
Lime juice	31.0	37.5	6.5	2.0
Simlin	14.7	17.4	2.7	2.5
Orange juice	69.0	74.0	5.0	3.4
Potato, dehydrated	99.4	111.0	11.6	5.0
Green pepper	169.0	173.0	4.0	5.2
Cauliflower	75.5	72.5	−3.0	6.3
Yellow squash	16.0	26.0	10.0	7.5
Parsley	209.0	219.0	10.0	8.3
Orange peeling	266.0	282.0	16.0	11.3
Grapefruit peeling	196.0	235.0	39.0	14.0
Kale	234.0	238.7	4.7	17.5
Broccoli	108.0	115.0	7.0	18.0
Cabbage, dehydrated	391.8	405.0	13.2	18.7
" "	512.0	540.0	28.0	22.5
Lemon peeling	204.0	210.0	6.0	25.0
Orange marmalade	21.2	23.0	1.8	2.4
" "	4.4	7.1	2.7	0.8
Apple butter	0.8	2.6	1.8	1.8

then diluted 2 to 5 times with metaphosphoric acid and glacial acetic acid to make a final solution containing 5 per cent metaphosphoric acid and 10 per cent acetic acid. This was passed through norit and the total dehy-

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droascorbic acid determined as previously described (2). The results are shown in Column 2 of Table I. The values for dehydroascorbic acid (Column 3) are obtained by subtracting each value of Column 1 from the corresponding value in Column 2. Their validity obviously depends upon the precision and specificity of the two methods used. The data of Column 4, Table I, were obtained by our direct procedure which will be described in detail below.

Direct Method for Dehydroascorbic Acid (for Plant Tissues Only)—The tissue is ground under usually 50 and never less than 20 parts of a solution containing 5 per cent metaphosphoric acid and 1 per cent thiourea. After filtration 4 cc. of extract are placed in each of three matched photoelectric colorimeter tubes. One tube is kept for a blank. To each of the others is added 1 cc. of 2 per cent 2,4-dinitrophenylhydrazine in approximately 9 N sulfuric acid. These are held at 37° for 3 hours, then cooled, together with the blank, in ice water. To each of the three tubes, while in the ice water bath, 5 cc. of 85 per cent sulfuric acid are added from a burette, a drop at a time, during not less than 1 minute. (Sulfuric acid, not grease, should be used for lubricating the stop-cock of the burette.) Finally 1 cc. of the 2 per cent 2,4-dinitrophenylhydrazine solution is placed in the blank tube. The tubes are shaken thoroughly under the ice water, removed to a rack, wiped dry after 30 minutes, and read in a photoelectric colorimeter, with a 540 mμ filter.

Colorimeter readings should be taken 30 to 45 minutes after removal from the ice bath. In some plant extracts a slight yellowish coloration appears when the 85 per cent sulfuric acid is added. This is compensated by the blank, to which the colorimeter is adjusted before the unknown is read. As the center setting does not remain constant in all cases, a greater increase in color sometimes taking place in the blank tube than in the unknown, the colorimeter readings should be made as directed above.

Appropriate standards must be run along with the unknowns, or, preferably, a calibration curve and chart are made with standard solutions of dehydroascorbic acid in concentrations ranging from 0.25 to 15 γ per cc. To prepare the dehydroascorbic acid standard, a solution of 25 mg. of ascorbic in 25 cc. of 5 per cent metaphosphoric acid is treated with 1 or 2 drops of bromine, shaken until yellow, decanted from the excess of bromine into a large test-tube, and aerated until colorless. Standards of appropriate concentrations are made by diluting with 5 per cent metaphosphoric acid containing 1 per cent thiourea.

DISCUSSION

Table I is of interest with respect to the methods for the determination of ascorbic acid. When little dehydroascorbic acid was present in the tissue,

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the results by the indophenol and the dinitrophenylhydrazine method are in good agreement. In other analyses, marked differences can be accounted for by the value obtained for dehydroascorbic acid by our direct method. The agreement between the data obtained by the widely different methods is evidence for the specificity of each, and suggests possibilities of obtaining more reliable and precise values for true ascorbic acid than can be secured by bioassay techniques.

Our studies indicate that the indophenol method, as modified by Bessey (3), is reliable for the estimation of ascorbic acid in most plant tissues, and

TABLE II

Recoveries of Dehydroascorbic Acid Added to Plant Tissues When Analyses Were Carried Out by Direct Dehydroascorbic Acid Method

The values are in terms of micrograms per 4 cc. of filtrates of 1:50 dilution.

Tissue	Amount in control filtrate	Amount added	Amount obtained after addition	Amount recovered	Per cent recovery
Lemon juice	1.8	34.2	38.4	36.6	107
Orange "	2.4	34.2	37.8	35.4	104
Cauliflower	3.3	34.2	39.4	36.1	105
Spinach	3.7	34.2	38.7	35.0	102
Kale	5.5	34.2	43.6	38.1	111
Apple	0.0	33.2	32.4	32.4	98
Peach	0.0	33.2	33.2	33.2	100
Pepper	6.1	37.2	47.0	40.9	110
String beans	1.3	37.2	39.4	38.1	102
Yellow squash	2.5	37.2	40.5	38.0	102
Simlin	3.5	37.2	41.4	37.9	102
Lemon peeling	17.2	29.7	50.0	32.8	110
Orange "	13.2	29.7	47.0	33.8	114
Lemon " *	3.3	25.8	30.7	27.4	106
Orange " *	4.6	25.8	31.3	26.7	103

* Filtrate of 1:200 dilution.

that the 2,4-dinitrophenylhydrazine method is satisfactory for the determination of both ascorbic acid and dehydroascorbic acid in the same material. The indophenol method is obviously inadequate when dehydroascorbic acid is present in appreciable amounts.

The recoveries obtained by our direct dehydroascorbic acid method, when dehydroascorbic acid was added to a group of plant tissues, are shown in Table II. The high recoveries in the first experiments with lemon and orange peeling (110 and 114 per cent) were due to the use of too low a dilution, 1:50. When these analyses were repeated with an extract diluted 1:200, the recoveries were satisfactory.

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One problem in vitamin C analysis is the possible interference in chemical methods by the reductone or enediol type of compounds that are formed by heating carbohydrate-containing foods in the presence of acid or alkali. These products rapidly decolorize indophenol (5) and also interfere in the dinitrophenylhydrazine methods. In Table III are summarized the results obtained by boiling carbohydrates in slightly acid, approximately neutral,

TABLE III

Apparent Vitamin C Produced by Boiling Carbohydrates in Slightly Acid, Approximately Neutral, and Alkaline Solutions

All solutions contained 1 gm. of carbohydrate per 100 cc.

Carbohydrate	Treatment	pH		Apparent ascorbic acid	Apparent ascorbic acid + dehydroascorbic acid	Apparent dehydroascorbic acid	
		Before boiling	After boiling	Indophenol method (1)	Dinitrophenylhydrazine method (2)	By difference (2) − (1)	Direct dinitrophenylhydrazine method
Glucose	Distilled H_2O solution boiled 4 hrs.	5.5	4.7	0.03	0.04	0.01	0.02
Fructose		5.9	4.4	0.00	0.08	0.08	0.04
Dextrin		3.7	3.7	0.00	0.04	0.04	0.003
Glucose	Phosphate-buffered solution boiled 4 hrs.	6.8	6.4	0.15	1.10	0.95	0.79
Fructose		6.8	6.6	0.14	1.19	1.05	0.79
Dextrin		6.8	6.6	0.01	0.20	0.19	0.21
Glucose	0.5% $NaHCO_3$ solution boiled 30 min.	8.3	8.5	13.1	20.0	6.9	4.9
Dextrin		8.3	9.2	2.4	3.4	1.0	1.6
Glucose	0.5% $NaHCO_3$ solution boiled 90 min.	8.3	8.4	23.4	20.6	−2.8	6.3
Dextrin		8.3	9.4	10.2	3.1	−7.1	2.8

and alkaline solutions. After 30 and 90 minutes boiling of alkaline solutions of glucose and dextrin, relatively large amounts of apparent vitamin C were obtained by each method, but when solutions of glucose, fructose, or dextrin were boiled for 4 hours at pH 5.5 to 3.7, only traces of substances that interfere in either of the analytical methods were produced; the same treatment of similar solutions buffered with phosphate at pH 6.8 also gave relatively low values.

It appears from the data of Table III that interference in analytical methods for vitamin C as a result of the cooking process is not serious when the heating is carried out, as is customary, in neutral or slightly acid solu-

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tions. In view of these results it is suggested that the higher values for vitamin C sometimes observed with the indophenol method with previously heated plant tissues are probably explained by the inactivation of the ascorbic acid oxidase of the tissue, with resultant preservation of the reduced form of the vitamin.

Adaptation of Original 2,4-Dinitrophenylhydrazine Method for Blood and Urine to Analysis of Plant Tissues—The 2,4-dinitrophenylhydrazine method of determining vitamin C was originally applied to blood and urine (2). As some laboratories have now adapted this method to other substances, we wish to make certain recommendations in regard to its application to plant tissues. Except for the need for a calibration curve and the use of a solution containing 5 per cent metaphosphoric acid and 10 per cent acetic acid instead of 6 per cent trichloroacetic acid for extracting the tissue, the procedure is essentially the same. Adsorption of the vitamin on the norit is counteracted by the presence of acetic acid. Trichloroacetic acid has the same effect, but in increased concentration it may crystallize out when the sulfuric acid is added later in the process, and so the use of acetic acid is preferable. Since the concentration of acetic acid influences the rate of coupling, the same amount of acetic acid must be used in preparing the calibration curve as in carrying out the analyses.

1 gm. of norit to 50 cc. of filtrate is recommended, but highly pigmented plant filtrates may have to be treated with norit two or three times to obtain complete clarification. When 10 per cent acetic acid was present in the extraction fluid, no decrease in value resulted when 3 gm. of norit per 50 cc. were added, but losses of 3.8 per cent were observed when 6 gm. were used. Filtrates containing 15 per cent acetic acid did not show diminished values when 8 gm. of norit per 50 cc. were added.

The sensitivity of the 2,4-dinitrophenylhydrazine methods requires the use of relatively dilute extracts, an advantage from the standpoint of interference from other substances. As a rule in making extracts we take an amount of tissue expected to yield a filtrate containing 0.2 mg. of vitamin in 100 cc. of filtrate, but this amount should not exceed 2 gm. per 100 cc. unless extremely small quantities of vitamin C are involved.

SUMMARY

1. A method for the direct determination of dehydroascorbic acid in the presence of ascorbic acid in plant tissues has been developed.

2. The dehydroascorbic acid of a metaphosphoric acid extract, in which the ascorbic acid is stabilized with thiourea, is coupled with 2,4-dinitrophenylhydrazine and the resulting derivative is treated with sulfuric acid to produce a red color which is measured photometrically.

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3. Certain modifications of the original 2,4-dinitrophenylhydrazine method for the determination of the total vitamin C in blood and urine have been made in order to adapt this technique to the analysis of plant tissues.

4. A comparison of results obtained with thirty-two plant tissues by the indophenol photometric and the 2,4-dinitrophenylhydrazine method has been made. Good agreement of results by the two methods was observed except when much dehydroascorbic acid was present.

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Jama 215:1671, 8 Mar 71

Interaction of Ascorbic Acid and Warfarin

To the editor.—In Sigell and Flessa's communication on "Drug Interactions with Anticoagulants" (214: 2035, 1970) they mentioned that ascorbic acid administration shortened the prothrombin time in animals receiving coumarin anticoagulants. I wish to report an example in which this held true in a human.

Report of a Case.—This 52-year-old nurse was hospitalized at University Hospital on Oct 1, 1970, because of pain in the chest aggravated by breathing. She also had pain in the right calf area which she had "bruised" one week previously. The pain in the chest came on suddenly after a brief exertion and was associated with shortness of breath.

Pertinent physical findings were as follows: blood pressure, 145/90 mm Hg; respirations, 24 per minute, pulse rate, 100 beats per minute and regular; temperature, 98.6 F. There were no wheezes or rales in auscultating the chest. The second heart sound was split with pulmonic second sound equal to aortic second sound. There were no murmurs present. The Homans' sign was positive on the right with calf tenderness.

The x-ray film of the chest and electrocardiogram were normal; however, the lung scan showed decreased perfusion in the right base. Lactic acid dehydrogenase level was twice the normal value with serum glutamic oxaloacetic transaminase and bilirubin levels within normal limits.

A diagnosis of pulmonary embolism secondary to phlebitis was made and the patient was started on a course of anticoagulation with heparin sodium. After nine days she was switched to anticoagulation orally with warfarin (Coumadin) sodium with gradual discontinuation of the heparin in the next two days. The patient was discharged on a regimen of warfarin sodium, 7.5 mg per day, and no other medication on the 17th hospital day, with her prothrombin time in the range of 23 seconds with a control of 12. The prothrombin time done weekly remained in that range until four weeks later when it dropped to 19, 17, and then 14 seconds with no response to an increase in the dosage of warfarin sodium to 10 mg, 15 mg, and 20 mg/day. She denied taking any other medications or changing her diet (such as, an increase in vitamin-K-containing foods) in any way. However, after the prothrombin time continued to stay low she was again questioned. It was found that she felt she was getting a cold, had heard ascorbic acid was good for that, and was administering ascorbic acid with the warfarin pills every morning. She had not considered this as a medication when questioned before. This was stopped and in two days the prothrombin time was 28 seconds with a control of 12.

Comment.—Since recently a Nobel prize winner has recommended high doses of ascorbic acid for prevention of the common cold and this has been highly publicized, we must be aware of its effect on other drugs. Also this case illustrates that when we question patients about medication, we must keep in mind that they may think only of prescription drugs.

George Rosenthal, MD
Roxbury, Mass

Byul. Eksp. Biol. Med. 62(8):96-98, 1966

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THE MECHANISM OF TERMINATION OF PREGNANCY BY ASCORBIC ACID

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One of the authors has previously shown [1-3] that large doses of ascorbic acid (AA) cause sexual disorders in guinea pigs, rats, and mice, which may result in the termination of pregnancy and in stillbirth. Termination of pregnancy by large doses of AA has also been observed in women.

The object of the present investigation was to study the mechanisms responsible for the sexual disturbances caused by AA and, in particular, to examine the role of the sex hormones in the termination of pregnancy.

EXPERIMENTAL METHOD

Two series of investigations were carried out. In series I, the cytological picture of vaginal smears of albino rats was studied after the animals had received estrogens and large doses of AA to terminate pregnancy.

In series II the excretion of estrogens, pregnandiol, and 17-ketosteroids was determined in women taking large doses of AA in order to terminate pregnancy.

The experiments were carried out on sexually mature female albino rats with a mean weight of 180 g. An ordinary diet was given. Smears were stained by Papanicolau's method. The animals were divided into three groups: the rats of group 1 (13) received an injection of folliculin in a dose of 25 i.u. per animal, dissolved in vegetable oil. The animals of group 2 (14) received AA, after preliminary neutralization, in a dose of 150 mg per animal. Both preparations were injected subcutaneously daily until the expected date of parturition or until the termination of pregnancy. The rats of group 3 (control, 17 animals) received subcutaneous injections of physiological saline.

To determine the effect of AA on the excretion of sex hormones in women, the compound was given in doses of 6 g daily for 3 days. The hormones in the urine were determined twice: once before administration of AA and again after the 3-day course. The excretion of the hormones was determined over a period of 24 h. The total estrogens were determined by Ingle's method, the pregnandiol by the method of Astwood and Johnson, as modified by Ordynets, and the 17-ketosteroids by Callow's method.

EXPERIMENTAL RESULTS

Of the 13 animals receiving folliculin (group 1), pregnancy was interrupted in 11 (on 8th-11th day of pregnancy). Two females gave birth to normal offspring at full term. Of the 14 animals receiving AA (group 2), pregnancy was terminated in 3 (on the 13th-15th day of pregnancy). Of the 17 females of the control group (group 3), normal offspring were born at full term, and two females were not pregnant.

At postmortem examination of the animals with interrupted pregnancy, either dead fetuses or signs of abortion were found in the uterus.

The cytological picture of the vaginal smears of the animals of the two experimental groups was more or less identical. Smears from females receiving folliculin showed an abundance of cells, mainly squamous epithelial cells with acidophilic staining properties, nucleated cells which were partly basophilic and partly acidophilic, and numerous leukocytes. In the smears from the animals receiving AA, the cell composition was the same as in those of the animals of group 1, except that fewer leukocytes were found. Before the interruption of pregnancy, the number of epithelial cells in the smears from the animals of groups 1 and 2 rose sharply, and the whole smear showed acidophilic staining properties. The smears from the animals of the control group contained very few cells. Among them were isolated nucleated cells and leukocytes. No epithelial cells were found. The staining of the films was basophilic.

P. M. Buiko Ukrainian Institute of Maternal and Child Welfare, Kiev. (Presented by Active Member of the Academy of Medical Sciences of the USSR A. P. Nikolaev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 62, No. 8, pp. 96-98, August, 1966. Original article submitted December 1, 1964.

Presumably the uniformity of the cytological picture of the vaginal smears of the rats receiving folliculin and AA may be interpreted as evidence of an increase in the estrogen background resulting from administration of AA.

Observations were made on twenty women aged from 20 to 40 years, seeking medical advice for termination of pregnancy in the case of a delay of 10-15 days in menstruation. Observations were made by the obstetrician-gynecologist L. I. Ivanyuta.

In 16 of the 20 women examined, bleeding of normal menstrual type appeared 1-3 days after a course of AA. In 4 women, bleeding did not take place. The excretion of estrogens after taking AA was increased in 12 of the 16 women examined (mean before taking AA 284 μg, after taking AA 360.4 μg). The excretion of pregnandiol and 17-ketosteroids remained practically unchanged after the course of AA.

It may be concluded from the results of these investigations that if large doses of AA are administered, the estrogen background is raised, and this may be a possible cause of the termination of pregnancy after taking large doses of AA.

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J. Nutr. 36: 205-213 1948

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THE RELATION OF ASCORBIC ACID METABOLISM IN THE RAT TO DIETS HIGH IN PROTEIN, CARBOHYDRATE OR FAT [1]

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Ascorbic acid deficiency has long been associated with disturbances in protein metabolism, particularly the formation of intercellular cementing substance. Sealock and Silberstein ('39) were the first workers to connect this deficiency with the metabolism of specific amino acids. The normal metabolism of certain amino acids is disturbed in scorbutic guinea pigs and immature infants (Levine, Gordon and Marples, '41; Christensen and Lynch, '48). Administration of ascorbic acid, often in large amounts, restores normal metabolism. In the present experiments we have attempted to determine whether ascorbic acid metabolism can be influenced in the rat by increasing protein metabolism while maintaining caloric intake constant.

Rats, therefore, were fed diets high in carbohydrate, fat or protein but in all cases adequate to maintain nitrogen balance, and the ascorbic acid concentrations in various organs determined.

METHODS

Adult male rats of the Sprague-Dawley strain were fed for 5 weeks by the stomach tube technique of Reinecke, Ball and Samuels ('39). The composition of the diets used is given in table 1.

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In addition, in experiment 2 the protein-fed rats were given 6 mg ascorbic acid per day. In experiment 3 the fat group and 1 protein group received 50 mg ascorbic acid per day by mouth.

At the end of the feeding period the rats were anesthetized with sodium amytal given intraperitoneally, 9 mg/100 gm

TABLE 1

Diets used in stomach tube feeding

	HIGH FAT	HIGH CARBOHYDRATE	HIGH PROTEIN
Lactalbumin, Labco (gm)	30	30	263.25
Gelatin, U.S.P. (gm)	30	30	30
Corn oil (gm)	105.8	3	3
Dextrin (gm)	0	233.25	0
Salt mixture, U.S.P. no. 1 (gm)	12.0	12.0	12.0
Oleum Percomorphum, reinforced (drops)[1]	12	12	12
Vitamin concentrate (gm)[2]	6.0	6.0	6.0
Water to make (ml)	900	900	900
Calories (per ml)	1.30	1.31	1.30
Caloric distribution (% of total calories)			
Protein	19.5	19.5	92.4
Fat	80	2.7	2.7
Carbohydrate	0.5	77.8	4.9

[1] Mead Johnson and Co.

[2] Lederle's Lederplex. According to label, 6 gm contained (in milligrams) thiamine hydrochloride 24, riboflavin 24, nicotinamide 120, pyridoxine 2.4, calcium pantothenate 36, choline 240, inositol 120 and folic acid 2.4; plus other water-soluble extractives from 96 gm liver.

body weight. The abdomen was then opened and blood withdrawn from the abdominal aorta. The various organs were removed, weighed, ground with sand and 6% trichloracetic acid and centrifuged. The supernatant solutions and blood plasma were analyzed for ascorbic acid by the method of Roe and Kuether ('43).

A sample of liver was also analyzed for total nitrogen by the Kjeldahl method.

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RESULTS

As can be seen in table 2, there were no significant differences in the ascorbic acid concentrations of any of the tissues when the fat-fed and carbohydrate-fed groups were compared, with the possible exception of the livers in experiment 1. In this case, when ascorbic acid was related to the nitrogen content of the livers, this difference was eliminated. Apparently a wide shift in the ratio of carbohydrate to fat as sources of calories did not affect ascorbic acid metabolism in a marked way.

On the other hand, the protein-fed groups showed significant differences from those on the other 2 diets. The ascorbic acid concentrations of muscle and blood were always lower in the animals receiving the high protein diet than in the other groups. The administration of high doses of ascorbic acid did not alter blood levels significantly, but the concentration in the muscles was definitely increased.

The kidneys and livers of protein-fed rats also had lower concentrations of ascorbic acid on the unsupplemented diets. Both organs, however, were larger in the animals on the high protein diets. When considered on the basis of ascorbic acid per 100 gm body weight, there was as much in the livers of the protein-fed rats as in the carbohydrate- or fat-fed groups; the lower concentration in the liver was offset by the larger size of the organ. This was not true in the case of the kidney; the concentration per 100 gm body weight was also significantly lower unless ascorbic acid was administered. The levels in both kidneys and liver were raised when the vitamin was fed.

As shown in table 3, the significant differences in total weight of organs are those which have been previously reported (Osborne, Mendel, Park and Winternitz, '25). The livers of the rats fed high protein were largest in experiment 2 where the caloric intake was also largest. In experiment 1, the livers of the fat-fed and protein-fed rats were larger than those of the rats fed carbohydrate but did not differ

TABLE 2

Ascorbic acid content of organs of male rats on high fat, high carbohydrate or high protein diets[1]

EXPERIMENT AND DIET	NO. RATS	LIVER			KIDNEY		BRAIN		ADRENALS		BLOOD	MUSCLE
		mg per gm Organ	mg per gm N	mg per 100 gm BW[2]	mg per gm Organ	mg per 100 gm BW[2]	mg per gm Organ	mg per 100 gm BW[2]	mg per gm Organ	mg per 100 gm BW[2]	mg per 100 ml	mg per gm Tissue
Experiment 1												
Fat diet	10	0.242 ± .017	7.84 ± 0.71	0.848 ± .052	0.153 ± .006	0.102 ± .005	0.252 ± .010	0.145 ± .005	3.19 ± .22	0.054 ± .0053	0.90 ± .04	0.037 ± .0019
Carbohydrate diet	10	0.262 ± .014	7.47 ± 0.53	0.723 ± .039	0.130 ± .010	0.084 ± .005	0.228 ± .019	0.137 ± .011	3.21 ± .14	0.052 ± .0037	0.94 ± .06	0.035 ± .0013
Protein diet	8	0.188 ± .010	5.27 ± 0.20	0.717 ± .047	0.068 ± .004	0.064 ± .004	0.227 ± .013	0.133 ± .008	3.19 ± .18	0.069 ± .0030	0.79 ± .05	0.026 ± .0010
Experiment 2												
Fat diet	5	0.289 ± .001		0.891 ± .022	0.137 ± .005	0.091 ± .004	0.368 ± .018	0.220 ± .011	3.86 ± .11	0.089 ± .0048	0.83 ± .06	0.025 ± .0003
Carbohydrate diet	7	0.337 ± .013		0.883 ± .052	0.143 ± .006	0.092 ± .004	0.380 ± .012	0.232 ± .011	3.31 ± .20	0.064 ± .0055	0.83 ± .06	0.025 ± .0008
Protein diet	5	0.222 ± .011		0.794 ± .048	0.088 ± .004	0.085 ± .005	0.334 ± .008	0.172 ± .008	3.89 ± .13	0.079 ± .0060	0.65 ± .07	0.019 ± .0006
Protein diet + 6 mg ascorbic acid injected	6	0.237 ± .024		0.928 ± .091	0.092 ± .007	0.092 ± .004	0.326 ± .022	0.183 ± .010	4.06 ± .13	0.083 ± .0033	0.64 ± .05	0.023 ± .0018
Experiment 3												
Fat diet + 50 mg ascorbic acid fed	7	0.331 ± .008		0.882 ± .042	0.221 ± .006	0.134 ± .006	0.323 ± .007	0.195 ± .008	3.79 ± .18	0.072 ± .0031	0.96 ± .04	0.031 ± .0018
Protein diet	7	0.278 ± .016		0.792 ± .038	0.131 ± .003	0.101 ± .002	0.308 ± .014	0.180 ± .011	3.57 ± .18	0.062 ± .0046	0.76 ± .06	0.024 ± .0010
Protein diet + 50 mg ascorbic acid fed	8	0.303 ± .015		0.808 ± .040	0.157 ± .007	0.124 ± .005	0.310 ± .006	0.180 ± .005	3.86 ± .16	0.064 ± .0026	0.80 ± .04	0.028 ± .0007

[1] All values are given as means with the standard errors of the means.

[2] Total body weight of animal at autopsy.

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TABLE 3

Weights of organs of rats on high fat, high carbohydrate, or high protein diets fed by stomach tube

DIET	RATS	CALORIES PER DAY	AVE. WT. AT START	CAL./100 GM/DAY	AVE. WT. AT END	LIVER [1]		KIDNEYS [1]	ADRENALS	BRAIN	THYMUS
						gm	*% N*	*gm*	*gm*	*gm*	*gm*
Experiment 1											
Fat diet	10	50.1	252	19.9	299	10.62 ± .46	3.30 ± .05	2.016 ± .081	0.050	1.726	0.214
Carbohydrate diet	10	48.0	249	19.3	285	7.91 ± .26	3.58 ± .05	1.878 ± .065	0.044	1.712	0.285
Protein diet	8	48.0	247	19.4	282	10.07 ± .30	3.72 ± .06	2.636 ± .101	0.050	1.676	0.211
Experiment 2											
Fat diet	5	48.0	296	16.2	291	8.97 ± .32	3.28 ± .20	1.944 ± .034	0.067	1.748	
Carbohydrate diet	7	48.0	295	16.3	292	7.61 ± .18	3.55 ± .03	1.884 ± .193	0.055	1.801	
Protein diet	5	55.0	304	18.1	338	12.14 ± .62	3.59 ± .03	3.269 ± .139	0.069	1.731	
Prot. and 6 mg ascorbic acid injected	6	52.0	286	18.2	318	12.28 ± .42	3.54 ± .06	3.183 ± .144	0.062	1.771	
Experiment 3											
Fat + 50 mg ascorbic acid fed	7	48.0	297	16.2	289	7.66 ± .29	3.59 ± .07	1.740 ± .053	0.056	1.732	
Protein diet	7	48.0	298	16.1	293	8.37 ± .37	3.84 ± .02	2.270 ± .077	0.050	1.700	
Protein + 50 mg ascorbic acid fed	8	48.0	308	15.6	296	7.97 ± .18	3.80 ± .04	2.364 ± .037	0.050	1.752	

[1] Values are given as means with the standard errors of the means.

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significantly between themselves. However, the nitrogen content of the livers of the protein-fed groups was always higher than that in the other groups of all 3 experiments. In experiment 1 where all rats received equicaloric rations in quantities sufficient to cause gains in weight, the rats receiving fat had accumulated enough lipid in the liver to offset the increased protein content of the livers of the protein-fed rats. In experiment 3 where the animals were slightly underfed, the livers tended to be smaller and to have higher nitrogen contents, but the difference between diets was still present.

The kidneys of the protein-fed rats were always larger. The difference was more marked if the calories on the high protein diet were sufficient for gains in weight, but it was still marked in experiment 3 where food intake was insufficient to maintain weight.

There was no significant difference in the size of the adrenals of the protein- and fat-fed rats. The average weight of the adrenals of the carbohydrate-fed animals was slightly smaller. These results confirm the observations of Ingle, Ginther and Nezamis ('43) that, in the adult rat, there is no significant increase in adrenal size on a protein diet. Since in these experiments 92% of the calories was obtained from protein it seems unlikely that the adrenals play any role in the catabolism of dietary protein; otherwise, some evidence of hypertrophy would have been expected.

DISCUSSION

There seems to be some effect of protein diet on the metabolism of ascorbic acid in the rat. While there is no definite evidence of the mechanism involved, it seems that the following hypothesis would most simply fit the facts:

As Sealock, Perkinson and Silberstein ('40) have shown, ascorbic acid is related to the metabolism of tyrosine and phenylalanine. It also appears to be involved in the metabolism of glutamine and glycine (Christensen and Lynch, '48). On the high protein diet used here relatively large amounts of these amino acids, as well as all others, were catabolized.

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In such a situation it might be expected that the utilization of ascorbic acid would be greatly increased. Production, in which the liver appears to play a prominent role (Smythe and King, '42), could not keep up with destruction, and concentrations in the plasma and in the tissues which would metabolize such amino acids decreased. In those tissues like the brain, which did not use these amino acids, or in those tissues where ascorbic acid appears to be associated with some other specific metabolic process, such as the adrenals, there was no significant effect of the diet.

When ascorbic acid was fed, the concentrations in the tissues where they were low were increased, but the vitamin was still removed so rapidly by these tissues that the plasma level did not rise significantly. This would be expected, since the distribution of ascorbic acid to the tissues is not simply a diffusion phenomenon, but must involve considerable energy utilization to maintain the marked concentration gradient. If there was an increase in the processes utilizing ascorbic acid it might be expected that there would be energy available for maintaining an increased concentration gradient. In no single tissue did this gradient reach the maximum for that tissue found by Kuether, Telford and Roe ('44) in guinea pigs.

On the other hand, if decreased production were primarily responsible for the low ascorbic acid values, one would have expected that the levels in all tissues, whether they utilized amino acids or not, would be decreased. This is seen in the scorbutic guinea pig where the levels in the brain and adrenals follow the general drop in tissue concentration. One would also have expected the circulating fluids to show the effect of dietary ascorbic acid, if tissue demand were normal and liver production alone decreased. Also, as Roberts and Spiegl '47) demonstrated, the production of ascorbic acid, like other functions of the liver, is dependent on the supply of sulphur-containing amino acids. These were not lacking in any of the diets, and certainly not in the high protein ration. A relationship between tyrosine catabolism and ascorbic acid has been

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demonstrated in liver and kidney tissues by Lan and Sealock ('44). It probably is also true for muscle since the aromatic amino acids undergo metabolism there. It seems most logical, therefore, to assume that the changes observed were due to increased destruction of ascorbic acid in the presence of high amino acid metabolism.

Heinemann ('36) studied the influence of a diet containing 50% of the calories as protein on the excretion of, and requirement for, ascorbic acid in the human being. A diet containing 11% of the calories as protein was used as the basis of comparison. He found no effect. Since our high protein diets contained 92% of the calories as protein the apparent difference in results may be due to the greater sustained protein level in our experiments. It may also be due to the different methods of study or to difference in species. Certainly there seems to be an influence of high protein diets on the tissue distribution of ascorbic acid in the rat.

CONCLUSIONS

A high protein diet led to a decrease in the concentration of ascorbic acid in tissues which metabolize considerable amounts of amino acids, such as the liver, kidneys and muscle. The levels in the plasma were also decreased.

There was no change in brain or adrenal ascorbic acid concentrations.

The enlargement of the liver on a high protein diet was sufficient to leave no significant difference in the amounts of liver ascorbic acid per unit body weight. This was not true of the kidney. This organ showed a marked increase in size on the high protein diet, but the concentrations of ascorbic acid were so low that there was less ascorbate per unit of body weight.

Administration of ascorbic acid led to an increase in the concentration of ascorbate in the liver, kidneys and muscle of protein-fed rats, but even doses of 50 mg a day did not significantly increase the concentration in the plasma.

It is thought that the results are best explained by an increased utilization of ascorbic acid in those tissues able to

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metabolize large amounts of amino acids. The muscle illustrates the changes in a tissue metabolizing both ascorbate and protein, the blood reflects the body balance, and the liver shows the compensation changes of a producing organ.

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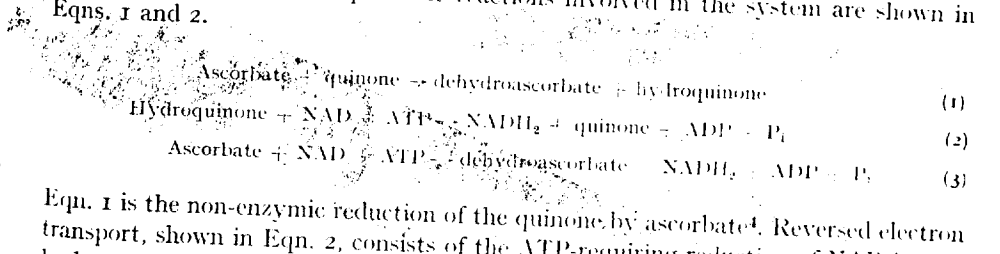
On the mechanism of oxidative phosphorylation IX. Energy-dependent reduction of nicotinamide adenine dinucleotide by ascorbate and ubiquinone or menadione

The reversal of oxidative phosphorylation originally observed by CHANCE AND HOLLUNGER[1] in mitochondria may now be considered a fully established phenomenon. Recent work on this system has been carried out using succinate as the electron donor and NAD as the acceptor in submitochondrial particles in the presence of added substrate amounts of ATP[2]. Both segments of the electron transport chain, one originating from succinate, the other from $NADH_2$, are implicated in this reaction. This communication describes a system of reversed electron transfer using ascorbate as electron donor and probably mediated only by the $NADH_2$ segment of the respiratory chain. Since ascorbate reacts with the electron transport chain very slowly[3], if at all, it was necessary to use an intermediate carrier which could transfer the electrons from ascorbate to the respiratory chain. Ubiquinone-5 (UQ_1 or Coenzyme Q_1) or menadione (vitamin K_3 or 2-methyl-1,4-naphthaquinone) proved to be satisfactory intermediate carriers. The probable reactions involved in the system are shown in Eqns. 1 and 2.



$$\text{Ascorbate} + \text{quinone} \rightarrow \text{dehydroascorbate} + \text{hydroquinone} \quad (1)$$

$$\text{Hydroquinone} + \text{NAD} + \text{ATP} \rightarrow \text{NADH}_2 + \text{quinone} + \text{ADP} + \text{P}_i \quad (2)$$

$$\text{Ascorbate} + \text{NAD} + \text{ATP} \rightarrow \text{dehydroascorbate} + \text{NADH}_2 + \text{ADP} + \text{P}_i \quad (3)$$

Eqn. 1 is the non-enzymic reduction of the quinone by ascorbate[4]. Reversed electron transport, shown in Eqn. 2, consists of the ATP-requiring reduction of NAD by the hydroquinone, via the respiratory chain.

In the routine assay, 0.05 M Tris sulfate, 3.3 mM $MgCl_2$, 1 mM NAD, 2 mg crystalline bovine serum albumin, 1 mM potassium cyanide, 0.02 M ascorbate, 0.1 mM menadione or 0.1 mM UQ_1 (added in 10 μl of methanol solution) and mitochondrial particles[5] (0.75 mg protein) in a final volume of 2.9 ml at pH 7.3 were incubated for 5 min at 30°. The reaction was started by the addition of 0.1 ml of 0.06 M ATP, and absorbancy readings at 340 mμ were taken every 2 min beginning with the first minute after the final addition. The inhibitor, when used, was present in the incubation

TABLE I

INHIBITION OF NAD REDUCTION BY UNCOUPLING COMPOUNDS AND OLIGOMYCIN

Experiments as in the text using menadione as the intermediate carrier.

Inhibitor	*mμmoles/min/mg*	*Inhibition (%)*
None	19.5	
2,4-Dinitrophenol, 0.1 mM	12.3	70
2,4-Dinitrophenol, 0.2 mM	0.0	100
None	12.9	
Dicumarol, 10 μM	1.7	90
Oligomycin, 0.1 μg/ml	15.4	94
Oligomycin, 0.3 μg/ml	0.0	100

medium. If it was added in ethanol solution, a similar amount of ethanol was used in the control assay. Each assay was carried out concurrently with a blank containing no ATP in order to correct for a slow, non-enzymic increase in absorbancy. The data are expressed as mμmoles of NAD reduced/min/mg mitochondrial particle protein.

In a preliminary communication[4], it was shown that both menadione and UQ_1 were equally efficient as intermediate carriers in the ATP-dependent reduction of NAD by ascorbate. The rate of NAD reduction was nearly the same as with succinate in the first 3 min and declined slowly. When ATP, quinone, ascorbate, NAD, or enzyme was left out of the medium, the rate of NAD reduction was quite small. The corrected increase at 340 mμ in the complete enzymic system was almost entirely due to NAD.

TABLE II

INHIBITION OF NAD REDUCTION BY ELECTRON TRANSPORT INHIBITORS

Experiments as in the text with menadione as the intermediate carrier.

Inhibitor	*mμmoles/min/mg*	*Inhibition (%)*
None	54.0	
Amytal, 0.5 mM	25.1	53
1.0 mM	10.5	81
1.5 mM	8.6	84
4,4,4-Trifluoro-1(2-thienyl)-1,3-butanedione, 0.05 mM	26.6	41
0.1 mM	1.2	48
Antimycin A, 1 μg/mg	51.9	4
10 μg/mg	39.0	28
15 μg/mg	27.1	50
None	34.2	
Rotenone, 0.05 μM	5.4	84
0.10 μM	2.3	93

reduction, since addition of lactate and lactate dehydrogenase resulted in a rapid decrease of absorbancy to nearly the original value. No careful search has been made for the phosphorylated NAD derivative reported to be accumulated under somewhat similar conditions[6].

The concentrations of ascorbate and menadione were selected for maximum rates. Nearly the same activity was obtained by reducing the concentrations of both to one-third the indicated levels. Serum albumin was included routinely since the activity was 30% higher in its presence. The reaction rate was optimal at pH 7.3; the decrease in activity on either side of it (from pH 6.8 to pH 8.5) was only slight. This contrasts with the rather high pH optimum of 8.5 in the energy-dependent NAD reduction by succinate[5,7].

The ATP-requiring reduction of NAD by ascorbate–menadione was inhibited almost completely by 2,4-dinitrophenol and dicumarol at concentrations which uncouple oxidative phosphorylation in mitochondria (Table I). Oligomycin, an inhibitor of energy-coupling reactions, also inhibited NAD reduction.

The respiration inhibitors, Amytal, rotenone and 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione also inhibited Reaction 3 (Table II) as in the ATP-dependent reduction.

NAD by succinate[2,5]. We have recently observed that rotenone strongly inhibits the oxidation of $NADH_2$ by UQ_{10}, both in particles and with a highly purified enzyme at similar low concentrations[9]. These results would implicate the $NADH_2$-UQ reductase (oxidoreductase) in the energy-dependent reduction of NAD by ascorbate–menadione.

NAD reduction by ascorbate–menadione was inhibited by antimycin, but only at unusually high levels (Table II), as was NAD reduction by succinate in the presence of added ATP[2,5] and $NADH_2$ oxidation by fumarate[5]. On the other hand, NAD reduction by ascorbate in the presence of tetramethyl-*p*-phenylenediamine in the same particles and under identical conditions was highly sensitive to antimycin (see ref. 10). Thus, two distinct effects of antimycin have been detected. Our results would suggest that reduced menadione reacts with an electron carrier located on the substrate side of the classical antimycin sensitive region (between cytochromes *b* and *c*).

Since 1.7 mM malonate had no effect on the system while the same level of malonate completely inhibited succinate-mediated NAD reduction in parallel experiments, the participation of the succinate dehydrogenase flavoprotein is excluded from the reaction.

These studies were carried out with the competent technical assistance of Mrs. C. WASHINGTON. UQ_1 was obtained through the courtesy of Dr. K. FOLKERS and of Dr. O. ISLER.

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Note added in proof: Contrary to our earlier preliminary report[9], it has been found that $NADH_2$ oxidation by UQ_{10}, catalyzed by the above particles or by the purified soluble $NADH_2$–UQ reductase, is not inhibited by trifluorothienylbutanedione. Since NAD reduction mediated by ascorbate–menadione or succinate, as well as aerobic oxidation of succinate, are inhibited in the same particles, it would appear that the point of entry of electrons from reduced menadione in the present experiments lies on the succinate-branch of the respiratory chain. (Received July 10th, 1964).

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THE EFFECT OF CHLORPROMAZINE, IPRONIAZID, AND CHLOROQUINE ON ADRENAL ASCORBIC ACID IN THE RAT

BY

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Various drugs have been shown to reduce the amount of ascorbic acid in the liver or the adrenal gland of the rat, or in both these organs. Hepatotoxic agents may or may not produce a decrease in the ascorbic acid content of the liver; thus the senecio alkaloid pterophine diminished the concentration of ascorbic acid in both the liver and the adrenal glands, whereas carbon tetrachloride produced little decrease in the liver and none in the adrenal glands (SAPEIKA, 1952). Among other drugs studied at the same time adrenaline and histamine depleted only the adrenal glands of ascorbic acid. Dicoumarol, which is not clinically regarded as a hepatotoxic drug, but which experimentally can cause centrilobular necrosis, produced a decrease in the ascorbic acid content of both organs in the doses used (SAPEIKA, 1953).

It was of interest to determine whether certain newer therapeutic agents would produce any change in the ascorbic acid content of the liver and the adrenals. The following drugs were investigated. Chlorpromazine hydrochloride, which has a central sympathetic suppressant action and peripheral anti-adrenaline action, may also occasionally produce jaundice associated with cholestasis in the liver. Iproniazid, which has a central nervous stimulant action, is an inhibitor of monoamine oxidase; it has produced jaundice and hepatic necrosis in a few patients (ZETZEL and KAPLAN, 1958). Chloroquine phosphate, which is a protozoicidal agent and is also used in rheumatoid arthritis, lupus erythematosus, and sun-sensitive patients, has a special feature in being concentrated in the liver in relatively large amounts without apparently causing liver damage.

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In view of the possible role of adrenaline, noradrenaline, and 5-hydroxytryptamine in the action of certain of these drugs the amines were also examined for their action on liver and adrenal ascorbic acid.

Methods

Equal numbers of male and nonpregnant female albino rats of the Wistar strain weighing 140-200 gm were used. They were fed the normal stock diet (Thomson, 1936). The drugs chlorpromazine, iproniazid, and chloroquine, were given in solution as a single dose through a stomach tube, the volume not exceeding 1 ml; in feeding experiments lasting ten days powdered stock diet containing a particular concentration of one of these drugs was given ad libitum. Noradrenaline, adrenaline and 5-hydroxytryptamine were given by subcutaneous injection. Control animals received stock diet only, or saline by injection.

At varying intervals after the beginning of the experiments pairs of animals were killed and their adrenal glands and 1 gm of freshly excised liver were weighed and assayed for their ascorbic acid content by the method of Kennaway, Kennaway and Warren (1944) and Kennaway and Tipler (1947) as in the previous investigations (Sapeika, 1952, 1953). The amounts of ascorbic acid are expressed as mgm per 100 gm of fresh tissue.

Results

In 16 control animals the values for liver ascorbic acid ranged from 21 to 30 (mean 25.5) mgm per 100 gm tissue, for the adrenal glands 300 to 420 (mean 364) mgm per 100 gm tissue. For all readings tissue from both a male and a female rat were examined together throughout the investigation.

Groups of 12 rats each fed a diet containing 0.05 per cent of chlorpromazine, iproniazid, and chloroquine examined every two or three days showed no loss of weight and no decrease in the liver and adrenal ascorbic acid over a period of ten days.

The effect of large single doses is described below.

Chlorpromazine hydrochloride. — A single oral dose of 50, 150, or 300 mgm per kgm body weight produced a decrease in the adrenal content of ascorbic acid for three to six hours as shown in Table I. There was no decrease in the liver content of ascorbic acid. Animals

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receiving the larger doses rapidly became very depressed and inactive but none died.

TABLE I

Adrenal ascorbic acid, mgm per 100 gm tissue

	½ hr.	1 hr.	2 hr.	3 hr.	6 hr.
50 mgm/kgm	300(2)	250(2)	287(4)	250(2)	304(2)
150 mgm/kgm	230(4)	233(4)	245(6)	250(4)	294(2)
300 mgm/kgm	250(2)	267(3)	260(5)	255(4)	336(3)

The figures in brackets indicate the number of animals used for the estimations.

Iproniazid. — Single doses of 50, 150, or 300 mgm per kgm produced no decrease in liver ascorbic acid, but the larger doses diminished the ascorbic acid content of the adrenal glands for approximately three hours, as shown in Table II.

TABLE II

Adrenal ascorbic acid, mgm per 100 gm tissue

	½ hr.	1 hr.	2 hr.	3 hr.	6 hr.
50 mgm/kgm	333(3)	308(2)	316(6)	330(2)	347(2)
150 mgm/kgm	260(3)	236(5)	284(5)	255(5)	350(2)
300 mgm/kgm	240(2)	283(6)	272(4)	316(4)	315(4)

Chloroquine phosphate. — A single dose of 300 mgm per kgm given orally produced no decrease in liver or adrenal ascorbic acid; one animal died one hour after receiving this dose but the remainder showed no ill-effects.

TABLE III

Adrenal ascorbic acid, mgm per 100 gm tissue

	½ hr.	1 hr.	2 hr.	3 hr.	6 hr.
300 mgm/kgm	290(2)	330(6)	345(4)	310(4)	306(4)

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Adrenaline, Noradrenaline, and 5-hydroxytryptamine. — These amines given by subcutaneous injection produced a decrease in the amount of ascorbic acid in the adrenals but not in the liver. The preparations injected were either Adrenaline hydrochloride (Parke-Davis and Co.), or Noradrenaline acid tartrate (levophed — Winthrop Products) or 5-hydroxytryptamine creatinine sulphate (May and Baker); in each case the dose of the preparation was 200 micrograms per 100 gm body weight.

TABLE IV

Adrenal ascorbic acid, mgm per 100 gm tissue

	½ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
Adrenaline	333(2)	287(2)	282(2)	277(2)	232(2)	236(2)
Noradrenaline	254(2)	237(4)	208(2)	223(4)	203(2)	200(2)
Hydroxytryptamine	264(2)	262(6)	231(4)	240(1)	228(2)	250(1)

DISCUSSION

The decrease in adrenal ascorbic acid produced by chlorpromazine, a central nervous depressant with sympathetic suppressant activity, is not easy to explain. In addition to blocking certain actions of adrenaline peripherally, but not that of noradrenaline, the drugs blocks certain effects of free 5-hydroxytryptamine although it does not release this amine (CARLSSON *et al.*, 1957).

Iproniazid rapidly enters the brain, liver, and other tissues, and causes inhibition of monoamine oxidase, which persists long after there are any detectable amounts of the drug in the tissues (HESS and WEISSBACH, 1958). The action of 5-hydroxytryptamine is prolonged in this way (SJOERDSMA *et al.*, 1955), (KABAKOW *et al.*, 1958). The effect of iproniazid in decreasing the adrenal ascorbic acid may therefore be an indirect one through a prolonged and combined action of adrenaline, noradrenaline, and 5-hydroxytryptamine; all three amines decrease the concentration of ascorbic acid in the adrenals but not in the liver. The action of 5-hydroxytryptamine may be direct or due to the release of adrenaline (REID, 1952), or to potentiation of adrenaline (COSTA and ZETLER, 1958), or to some extent to the release of histamine e.g. from the skin (FELDBERG and SMITH, 1953), or through the release of corticotrophin (PAGE, 1958).

Chloroquine is a drug that accumulates in high concentration in the

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liver. It has been administered for a few days in malaria, for longer periods in hepatic amoebiasis, and for several weeks in rheumatoid arthritis. In the present investigation the large doses used did not decrease the ascorbic acid content of the liver or the adrenal glands.

Summary

Chlorpromazine and iproniazid administered orally in single doses to rats produce a decrease in the ascorbic acid content of the adrenal glands but not of the liver. Chloroquine produces no such decrease.

Adrenaline, noradrenaline, and 5-hydroxytryptamine administered subcutaneously produce a decrease in the adrenal but not in the liver ascorbic acid. The possible role of these monoamines in the action of iproniazid is considered.

My thanks are due to Mr. J. W. Bates for assistance with the estimations, and to the Staff Research Fund of the University of Cape Town for defraying expenses in this investigation.

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Oxidimetric determination of ascorbic acid with potassium hexacyanoferrate(III) in acid medium

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ACCORDING to Kolthoff and Belcher[1] hexacyanoferrate(III) is obtainable in a high state of purity and serves as its own primary standard. It is a convenient oxidimetric reagent because of its stability in aqueous solution and its large equivalent weight. It is mostly employed for the determination of organic substances in neutral or alkaline media. It has been used for the determination of ascorbic acid by Hubicka[2] at pH 7, employing an amperometric technique and by van Pinxteren and Verloop[3] with a dead-stop end-point procedure in bicarbonate medium. Erdey and Svehla[4] proposed the visual titration of ascorbic acid with this reagent in a slightly alkaline medium buffered with sodium acetate, using 2,6-dichlorophenolindophenol as redox indicator. In an attempt to determine the redox potentials of the ascorbic acid $\rightleftharpoons$ dehydroascorbic acid system from potentiometric titration curves, Ball[5] reported that at pH 5 not only ascorbic acid but also dehydroascorbic acid reduces hexacyanoferrate(III). Abramov and Kadyrov[6] proposed the indirect determination in a slightly acid medium. After an aliquot of the ascorbic acid solution was reacted with a known and excessive amount of hexacyanoferrate(III) in the presence of zinc sulphate, the unreacted oxidant was treated with an excess of potassium iodide and the iodine liberated titrated with sodium thiosulphate, with starch as indicator. Sant[7] improved the method by direct titration in a medium buffered with zinc acetate to pH 6, with a starch-iodide end-point. In both cases, the zinc salt raises the redox potential of the hexacyanoferrate system towards the end-point by precipitating the hexacyanoferrate(II). At the higher pH the titration is likely to be vitiated by the reaction of dehydroascorbic acid with the oxidizing titrant, as stated by Ball. We have, therefore, undertaken this investigation to ascertain the conditions under which potassium hexacyanoferrate(III) can be used for the direct titration of ascorbic acid in acid medium with visual and potentiometric end-points.

EXPERIMENTAL

Reagents

Potassium hexacyanoferrate(III) solution, 0·1*N*. Stored in an amber bottle, and diluted as required.

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Ascorbic acid solution, 0·1*N*. Stabilized with a small amount of sulphuric acid, and standardized against potassium iodate. Diluted as required and standardized with iodate.

Demineralized water was used throughout because of the decomposition of ascorbic acid by traces of metallic impurities.

Visual titration in sulphuric acid medium

Oxidation of barium diphenylaminesulphonate by hexacyanoferrate(III) is rapid only when the sulphuric acid concentration is greater than 4*M* and reduction of oxidized indicator by ascorbic acid is rapid at all sulphuric acid concentrations <5*M* but retarded at concentrations >5*M*. For this reason the range of acidity for titration should be 4–5*M* at the equivalence point. To 40 ml of 8*M* sulphuric acid in a 250-ml conical flask add 3–8 ml ascorbic acid solution and 1 drop of 0·2% solution barium diphenylaminesulphonate and titrate with hexacyanoferrate(III). The equivalence point is heralded by transient appearance of a violet colour. Towards the equivalence point, the titrant is added dropwise at 3–4 sec. intervals. The end-point is indicated by a violet colour stable for 2–3 min. At low ascorbic acid concentration (20–200 μg/ml) the consumption of hexacyanoferrate(III) is low by 1–2% because of aerial oxidation of the ascorbic acid, but the results are accurate if a carbon dioxide atmosphere is used and an indicator correction of 0·02 ml of 0·01*M* titrant is deducted. No inert atmosphere is necessary for more concentrated solutions. Typical results are given in Table I.

TABLE I.—TITRIMETRIC DETERMINATION OF ASCORBIC ACID WITH HEXACYANOFERRATE(III)

Amount of ascorbic acid, *mg*			
Taken	Found	Taken	Found
72·74	72·70	8·166	8·167
57·73	57·61	7·705	7·691
48·21	48·39	6·742	6·751
43·42	43·25	5·780	5·792
36·38	36·47	5·154	5·140
25·14	25·18	3·992	3·987
		3·280	3·284
		1·919	1·920

Interferences

A tenfold excess of oxalic, citric, tartaric, succinic, malic and acetic acids, and glucose, fructose and sucrose does not interfere.

Visual titrations in other acidic media

Similar results are obtained in 7–10*M* hydrochloric acid or 9·0–11·0*M* phosphoric acid. The end point is sluggish (10–30 sec between additions) in phosphoric acid medium, but an advantage is that even dilute solutions of ascorbic acid (0·005*M*) can be titrated in air.

TABLE II.—POTENTIOMETRIC TITRATION OF ASCORBIC ACID WITH HEXACYANOFERRATE(III) IN 10*M* PHOSPHORIC ACID

Amount of ascorbic acid, *mg*	
Taken	Found
24·13	24·22
29·44	29·44
38·71	38·55
48·78	48·78
58·02	57·76
66·95	66·95
73·55	73·47

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Potentiometric titrations

For potentiometric titration a bright platinum rod is used as indicator electrode and a saturated calomel cell as reference electrode. In 4·5–5·5*M* sulphuric acid medium the potential stabilizes within 1 min in the early stages and 5 min at the end-point. The potential break is 100–120 mV per drop (0·04 ml) of 0·1*N* hexacyanoferrate(III), but the end-point is 1–2% lower than the true equivalence point. However, with rapid titration in an inert atmosphere and immediate reading of potential in the early stages and a wait of 1 min before readings near the end-point, an inflexion occurs at the true equivalence point. This implies that in a slow titration some aerial oxidation occurs.

Potentiometric titration in 10–12*M* phosphoric acid gives an end-point that corresponds to the true equivalence point and there is no need to maintain an inert atmosphere. Table II shows the relative error is 0·4%. The results are not affected by the presence of a 10–20 fold excess of sucrose, dextrose, citric acid, tartaric acid, malic acid and oxalic acid.

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Summary—Conditions have been developed for the titrimetric determination of ascorbic acid with hexacyanoferrate(III), with potentiometric and visual end-points, in sulphuric, hydrochloric or phosphoric acid media. Several organic substances likely to be present in plant tissues do not interfere.

Zusammenfassung—Die Bedingungen zur titrimetrischen Bestimmung von Ascorbinsäure mit Hexacyanoferrat(III) mit potentiometrischer oder visueller Endpunktsbestimmung in schwefelsaurem, salzsaurem oder phosphorsaurem Medium wurden ermittelt. Mehrere organische Substanzen, die in Pflanzengeweben wahrscheinlich vorkommen, stören bei der Bestimmung nicht.

Résumé—On a mis au point les conditions du dosage titrimétrique de l'acide ascorbique au moyen d'hexacyanoferrate(III), avec points de fin de dosage potentiométrique et visuel, en milieux acide sulfurique, chlorhydrique ou phosphorique. Plusieurs substances organiques susceptibles d'être présentes dans les tissus végétaux n'interfèrent pas.

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Microtitrimetric determination of uranium in nitric acid medium in the presence of excess of iron, magnesium and aluminium

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Davies and Gray[1] developed a method for titration of uranium(VI) with iron(II) in phosphoric acid medium after reduction to U(IV) and eliminated the interference of nitrate. Their method also avoids the sluggishness of reaction near the end-point that is experienced with the Rao and Sagi method.[2] The Davies and Gray procedure was developed for 200–300 mg amounts of uranium, and has since been

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EFFECT OF SUCCINIC ACID, PARGYLINE OR L-ASCORBIC ACID ON BRAIN GAMMA-AMINOBUTYRIC ACID (GABA): A MECHANISM OF PROTECTION AGAINST HYPERBARIC OXYGEN (OHP) TOXICITY.

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Since decreased levels of brain GABA have been implicated in OHP toxicity, we investigated the effect of pargyline, succinate or ascorbate on brain GABA in relation to OHP toxicity. IP administration of pargyline (100mg/kg), succinate or ascorbate (12 mg/kg) provided protection against OHP toxicity (convulsions, pulmonary edema and hemorrhage, post-exposure mortality). Succinate or pargyline elevated GABA in unexposed mice and prevented the GABA depletion in OHP exposed mice. Ascorbate was without effect in either group. In addition, there was a significant correlation between OHP seizure susceptibility and brain GABA in succinate or pargyline treated mice. It is suggested that the protective effect of succinate or pargyline is mediated through effects on GABA pathway. Protection by ascorbate is through an alternate mechanism. (Supported by PHS grant 1-T01 ES 00104-03 through the Institute of Environmental Biology.)

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THE RELATIONSHIP OF VITAMIN C AND ACTH IN EXPERIMENTAL WOUNDS

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THE PURPOSE of these experiments was to evaluate the relation of ACTH and vitamin C in wound healing. The importance of vitamin C in the production of collagen in healing wounds is well known, being conclusively demonstrated by Wohlbach and Howe many years ago. Ragan and his associates, Spain, and others have recently indicated the delaying effects of cortisone and ACTH on wound healing in rabbits and mice. At the same time Sayers has shown that there is a depletion of vitamin C in the adrenals of rats following the administration of ACTH. Many have demonstrated the remarkable clinical effects of ACTH and cortisone in the collagen diseases. It seemed logical to postulate that the inhibition of fibroplasia and collagen formation in wound healing might be related to depletion by ACTH of the vitamin C stored in the body. Indeed, recently several writers, on more or less empirical grounds, have stated that vitamin C should be given in conditions involving a stress reaction or during the administration of ACTH. Stepto has recently suggested a relationship between ascorbic acid intake and ACTH activity of the pituitary. The results of these studies, though failing to demonstrate a significant effect of ACTH on wound healing, fully confirm the observation that vitamin C is the most important dietary factor in promoting fibroplasia and collagen formation in healing wounds. Vitamin C levels in blood serum showed minimal irregular fluctuations during phases of wound healing and ACTH administration. In tissue juice from the healing area, fluctuations in vitamin C concentrations were much greater and seemed to follow a pattern irrespective of the administration of vitamin C or ACTH, or serum levels of vitamin C.

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METHODS

Male guinea pigs weighing 400 grams were used in order to eliminate the corpus luteum as a potential source of vitamin C. The animals were randomized and placed in individual metabolism cages and fed a standard Rockland diet fortified with 20 milligrams of vitamin C per 100 grams. Other animals were rendered scorbutic on a standard Sherman diet. Fibroplasia was assayed in three different ways. First, the amount of growth in and around a subcutaneously implanted hollow tantalum mesh sphere or cylinder, illustrated in a previous article (11), was recorded grossly and studied microscopically using, routinely, hematoxylin and eosin stain and Mallory's aniline blue stain for collagen. Fibroblasts were also placed in tissue culture to observe, if possible, any variations in growth characteristics in vitro utilizing methods described in an earlier publication (11). Secondly, a standard wound was made on the dorsum of the animals by excising a circle of skin and subcutaneous tissue according to techniques described previously (8), through the panniculus carnosus measuring 2.5 centimeters in diameter. The animals were randomized and the wounds photographed at a constant distance and magnification every third day until closure. The photographs of the wounds were traced with a planimeter. Thirdly, 3 simple linear incisions, 3 centimeters in length, were placed on the dorsum of the guinea pigs extending through the panniculus carnosus. These wounds were sutured with 0.003 inch tantalum wire. After 9 days the sutures were carefully removed, the animals sacrificed, and strips 1 centimeter in width were cut through the wound by means of 2 chisels welded 1 centimeter apart. These

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TABLE I.—TENSILE STRENGTHS OF INCISED WOUNDS AT 9 DAYS—EXPERIMENT 1

Scorbutic control		150.8 ± 52.4
Vitamin C (10 mgm.)	405.0	
Vitamin C (50 mgm.)	421.8	
ACTH plus vitamin C (10 mgm.)	473.0	418.2 ± 13.1
ACTH plus vitamin C (50 mgm.)	373.0	

Each value represents an average of 3 wounds on 6 animals. Note marked effect of scurvy. Large amounts of vitamin C have no greater benefit than smaller doses. No significant effect of ACTH.

TABLE II.—TENSILE STRENGTHS OF INCISED WOUNDS AT 9 DAYS—EXPERIMENT 2

Scorbutic control	163.2 ± 24.9
Vitamin C	318.7 ± 24.9
ACTH	140.5 ± 22.7
ACTH plus vitamin C	339.0 ± 22.7

Each value represents an average of 3 wounds on 5 animals. The variations are related to the presence or absence of vitamin C with no specific effect of ACTH.

strips were then placed in a tensiometer and tensile strengths determined by using a constant drip of mercury as indicated in Figure 1. Bursting strengths using a Mullen paper tester proved unsatisfactory. In addition to assaying fibrosis and wound healing by the methods described, vitamin C levels were determined quantitatively by the method of Lowry and associates in the blood serum and in tissue fluid secured from within tantalum mesh cylinders during the first 3 weeks of the encapsulating fibrotic process of repair around the mesh. In these experiments 6 groups of animals were used in varying combinations. One served as standard "controls" receiving Rockland guinea pig chow, the second was scorbutic, the third received supplementary vitamin C either by mouth or injection, the fourth received ACTH, the fifth was scorbutic plus ACTH, and the sixth received vitamin C and ACTH. The dosage of ACTH was 0.005 gram intramuscularly twice a day. Vitamin C was given orally or by subcutaneous injection once a day in 0.010 or 0.050 gram amounts

RESULTS

In the first series of experiments, using 90 guinea pigs and employing the subcutaneously implanted mesh, the groups receiving ACTH consistently had a more hemorrhagic reaction than the controls during the first 10 to 12 days, but after this they were indistinguishable from the controls. The animals receiving both

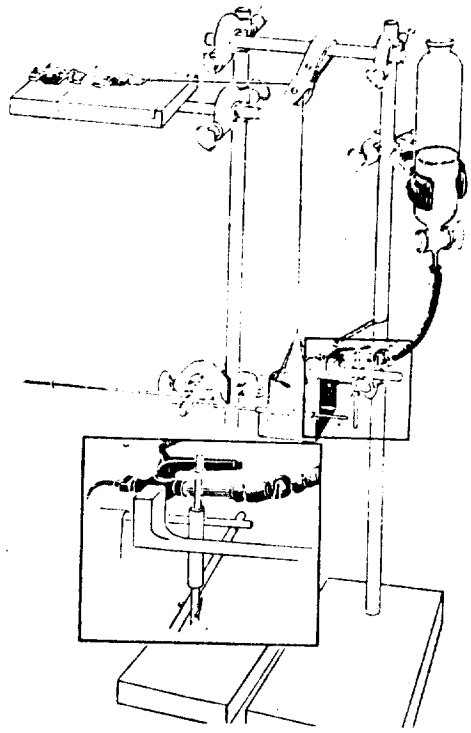

Fig. 1. Diagram of tensiometer utilizing a constant mercury drip and 1 centimeter strips from linear wounds on the dorsum of the guinea pig. When the strip breaks, the mercury collector drops, lowering the bar, allowing spring closure of the 3 way stopcock.

ACTH and vitamin C consistently showed the least gross reaction of any group. Fibrosis was maximal, and followed the pattern described earlier (11). The scorbutic animals, though failing to develop normally, had a marked hemorrhagic reaction in and around the mesh up to 30 days. Although there was, at this time, some decrease in the reaction, there was little adherence of the mesh to the bloody granulation tissue, and the wounds separated easily. The microscopic features of the reactions were essentially the same in all groups except the scorbutic series in which fibrosis was markedly decreased and very little ground substance or collagen was laid down. There was the suggestion that fibroblasts from animals receiving ACTH grew with less vigor in tissue culture than those from "normal" animals or from animals supplemented with vitamin C. This observation is being studied specifically in further experiments.

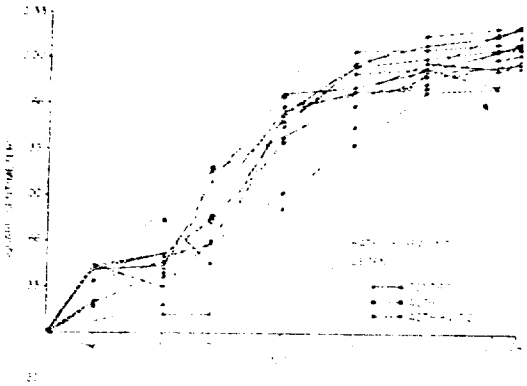

Fig. 2. Wound closure of three groups of guinea pigs as measured by planimeter tracings of photographs of wounds, expressed as square centimeters. No significant differences in rates of closure were noted in any of the treatment groups.

In the second type of experiment, the rate of wound closure was determined in a series of 15 guinea pigs divided into three groups as indicated in Figure 2. No significant difference was observed in any of the groups. Closure was not delayed by ACTH nor was it accelerated by the addition of vitamin C.

In the third type of experiment the tensile strengths of simply sutured linear wounds were measured. The guinea pigs were arranged in 4 or 5 treatment groups using 6 animals for each group (Tables I and II). The data indicated that ACTH did not reduce tensile strengths and that large amounts of vitamin C did not increase tensile strengths beyond that of small amounts. There was the suggestion of some interaction between vitamin C and ACTH with slight reduction of tensile strengths

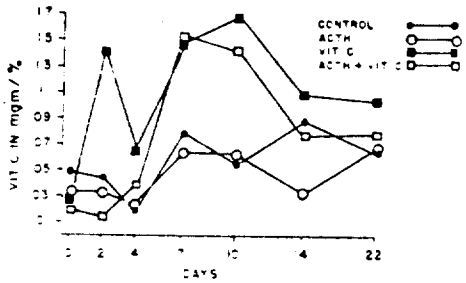

Fig. 5. Vitamin C concentrations of blood serum (experiment [illegible]) of guinea pigs following implantation of tantalum mesh cylinders. Variations are related to vitamin C intake and ACTH. Fifty milligrams of vitamin C [illegible] administered subcutaneously daily.

with ACTH administration, but it did not prove to be statistically significant. In all groups the scorbutic state was associated with a very low tensile strength. The data concerning vitamin C levels in the blood serum and tissue fluid of guinea pigs during the first 3 weeks of repair are summarized in Figures 3, 4, and 5. Each point represents the average of duplicate determinations on specimens from 5 animals. Several trends are indicated. The tissue fluid exhibits definite fluctuations in vitamin C concentration that were reproducible and were quite independent of the serum level. These variations in vitamin C concentration may be quite significant and may represent different phases or velocities of the reparative processes. When the animal was scorbutic the tissue fluid concentration of vitamin C was higher than serum level. When the animal received excessive amounts of vitamin C the levels in the tissue fluid were generally lower than in the serum. ACTH seemed to have little effect on these vitamin C levels in blood serum or tissue juice. Thus the wound fluid may contain vitamin C levels very different from those of serum despite remarkable similarities of electrolyte and electrophoretic protein patterns in the two fluids.

DISCUSSION

The findings seem definite, namely: in the guinea pig ACTH does not seem to delay wound healing significantly. Grossly ACTH seemed to produce a greater reaction about the tantalum mesh spheres during the first days of encapsulating fibrosis, resembling a mild scorbutic state. The lack of vitamin C resulted in marked bloody granulation tissue with little fibroplasia and collagen formation, and a marked reduction in tensile strengths. There seemed to be no significant relation between ACTH and vitamin C in wound healing under these experimental conditions. In another unrelated group of experiments in which cortisone was used instead of ACTH no significant effect of cortisone could be observed. It should be stressed that only guinea pigs were used and that certain caution should be used when applying results to man. The guinea pig was chosen because they, like man, do not synthesize vitamin C and can be ren-

dered scorbutic. In a recent abstract an almost identical experiment is reported by Pirani and associates. Studying linear abdominal wounds histologically, normal guinea pigs fortified with vitamin C showed greater healing, fibrosis, and collagen formation than unfortified animals on a standard adequate diet. ACTH inhibited collagen formation and fibroplasia slightly on the fifth postoperative day in the normal controls but had no effect on either the scorbutic or fortified animals. The adrenal response was not modified significantly by variations in the vitamin C intake. They concluded that the role of ascorbic acid in wound healing is a direct one and not mediated through the adrenal cortex.

The clinical implications from these experiments are that the stress response of a surgical procedure has little effect on local wound healing in the absence of other metabolic alterations. The carefully controlled reports by Localio (4, 5) on reduction of tensile strengths in wounds in rats during pregnancy, following multiple fractures, and massive doses of ACTH should be mentioned here. However, these measurements were made during only one phase of the healing process, the fifth day, when fibroblastic activity is maximal, but when the actual number of fibroblasts is low and collagen formation minimal because of the time lag. At 10 days no differences could be noted. Recent literature is rather contradictory in regard to the delaying effects of ACTH and cortisone in wound healing. A portion of this literature has been reviewed by Findlay in a paper indicating that cortisone inhibition of wound healing is increased by hypoproteinemia and debility. Care should be exercised in considering the various conflicting experimental and clinical results. Species variation has already been alluded to. Very few observers consider the entire healing cycle of a wound. There is tremendous variation in the relative biologic dosage levels of the corticoids, without due consideration of the state of the adrenals. Lastly, in clinical experience, the coexistence of metabolic disease, dietary variations, and poor liver function with impaired protein synthesis has lead to possible error in interpretation of data relating to wound healing.

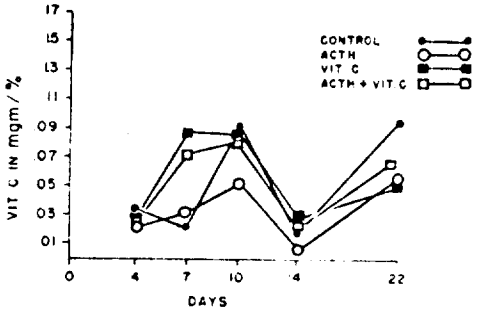

Fig. 4. Vitamin C levels in tissue fluid (experiment 1) within subcutaneously implanted tantalum mesh cylinders. Note fluctuations and variation from serum levels (Fig. 3). A slightly lower level of vitamin C in the ACTH treated group may be significant.

The monumental efforts of previous investigators is emphasized, namely: that vitamin C is essential for wound healing, which according to our concepts is essentially fibroplasia and collagen formation, with surface regeneration of epithelium, mucosa, mesothelium, or endothelium, depending on the site of the wound. Large amounts of vitamin C are nontoxic and seem indicated in all cases of major surgery though it is recognized that the scorbutic state is quite rare in the human subject. In a recent paper, Crandon has reported interesting studies relating to wide tissue variations in vitamin C concentration in the buffy coat, whole blood, serum, muscle, and fascia in surgical patients. Despite the wide ranges of values, clinical scurvy was rare, though when present it resulted in wounds that failed to heal. Bartlett and associates have determined the vitamin C content of

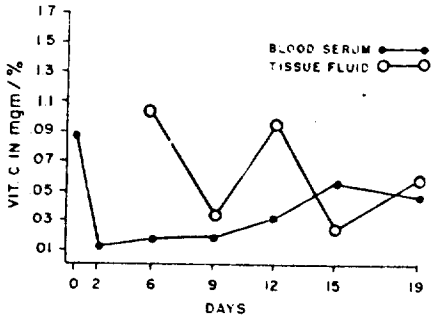

Fig. 5. Vitamin C concentrations of blood serum and tissue fluid of scorbutic controls (experiment 2). Note fluctuations of vitamin C concentrations of tissue fluid with higher concentration than serum.

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healing wounds and adjacent or distant muscle in guinea pigs receiving high or low intakes of vitamin C. Animals on a scorbutic diet showed essentially no increase in ascorbic acid in the healing area. With a high ascorbic acid intake there were striking increases of vitamin C in the healing wound compared with distant or control muscle tissue. Our data indicate a rather constantly higher level of vitamin C in the tissue fluid of healing areas than in the serum of the scorbutic animal. The vitamin C level in the tissue fluid showed reproducible fluctuations during the healing cycle that were independent of serum levels. This supports our present concept of the basic autonomy of a wound and its constant biologic behavior in the absence of infection with adequate vitamin C and protein stored in the body.

SUMMARY

1. Using male guinea pigs, fibrosis and wound healing was assayed by the following techniques: a subcutaneously implanted tantalum mesh cylinder, wound closures, and tensile strengths of primarily closed linear wounds.

2. Vitamin C concentrations were determined in blood serum and in tissue juice within the tantalum mesh cylinders during the first 3 weeks of the fibrotic encapsulating and penetrating process following subcutaneous implantation.

3. Six treatment groups of animals were employed in different combinations: control, scorbutic, ACTH, vitamin C, ACTH plus vitamin C, and scorbutic plus ACTH.

4. There was no significant effect of ACTH on the healing process or relation between ACTH and vitamin C in healing wounds.

5. Vitamin C seemed to be the most important single substance in fibrosis and collagen formation.

6. Vitamin C levels in blood serum showed moderate irregular fluctuations during phases of wound healing and ACTH administration.

7. In tissue juice from tantalum mesh cylinders, fluctuations in vitamin C concentrations were much greater and seemed to follow a pattern independent of the administration of vitamin C or ACTH or blood serum levels of vitamin C.

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IV. THEORETICAL AND EXPERIMENTAL MEDICINE

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From the research laboratories for experimental oncology (Director: Dr. med. habil. F. Schmidt) and from the Institute for Nutrition (President: Prof. Dr.Dr. Dr. K. Lohmann) of the German Academy of Sciences at Berlin in Potsdam-Rehbrucke.

VITAMIN C AND CANCER

by F. Schmidt, H. Schmanke and B. Gassmann

Vitamin C, of all vitamins, has hitherto found the greatest interest in connection with malignant tumors. One basis for this may be found in the fact that this vitamin before all others occurs in the highest concentration, absolutely and relatively, in tumor tissue. A series of publications also verifies certain successes of therapy with vitamin C in malignant growths. However, the study of the literature yields so many contradictory and deviating findings that we thought it time to subject some partial questions on this theme to renewed experimental testing.

We examined especially the content of different transplant tumors in rats for L- ascorbic acid (AS) and dehydroascorbic acid (DAS) in comparison with parenchymatose organs of tumor-infected and normal animals. The DAS moreover claimed our highest attention because monodehydroascorbic acid according to the new concepts (101) serves as the proper effective form of vitamin C and because the view is advanced that DAS occurs in tumors only in extremely small concentrations (53, 80). Remarkably however on just this last question no experimental findings exist, as a review of available literature showed.

In some comparative experiments we also further tested tumors and normal tissue of guinea pigs for AS and DAS content, because better possibilities for comparison with human data exist here, since as is known the guinea pig, unlike the rat, cannot synthesize its own vitamin C.

In some extensions of the tests we further examined the influence of the addition of higher doses of vitamin C on statistical occurrence and growth of some transplantable guinea pig sarcomas.

Experimental Materials and Methods

In the experiments on rats our own laboratory-grown animals were used, who received the same food of our own production (159).

Rats with the following transplantation tumors were investigated: Walker-Carcinosarcoma 256, Jensen-sarcoma, Yoshida-sarcoma, ES 4-sarcoma (60), Chlorom (Shay). The tumor animals belonged to different stages: Initial stage: tumor about 3 g., Advanced stage, tumor about the size of a hen's egg. The experimental material was cleaned of macroscopically visible necroses as far as possible; in the advanced tumors only the marginal parts were used. Altogether we examined 57 tumor rats, aside from tumors of the liver, spleen, testicles and brain, while in the normal control rats we also examined the kidneys.

In the test series on guineapigs the Daels-sarcoma was used.[1]

The therapeutic experiments on guinea pigs were carried out in 5 series. In these the animals of each experimental group got 50 mg daily of "Ascorvit forte" (VEB Jenapharm) by mouth or intramuscular injection

[1] We thank Professor Dr. Thurzo, Oncological Institute of Bratislava, for this strain.

beginning 3 days before the intramuscular transplantation of the Daels-sarcoma.

For determination of the AS and DAS the tumor and organ tissues immdeiately after removal were placed in ice water or frozen in a stroage refrigerator , in case vitamin C determination could not be made at once. After weighing there followed a fine reduction with a homogenizer (Ultraturrax, Fa. Janke & Kunkel KG, Staufen i. Br.) in 2 % oxalic acid.

For determination of albumin 10 ml of 10% trichloroacetic acid was added and filled up to 50 ml with 2 % oxalic acid. After filtration the AS contained in the sample was oxidized according to the m experimental method of W. Feldheim (52) with surplus 2,6 dichlorophenolindophenol solution. By extraction of the excess coloring material with amyl acetate the oxidation was interrupted after 15 and 30 seconds and the unconsumed quantity of the oxidation medium photometrically determined. On the basis of the simple assumption that the reactants consume the same quantity of 2,6 dichlorophenolindophenol in the time from 0 to 15 seconds as in the time from 15 to 30 seconds, the consumption by reactants (glutathion,, reductone, redctive acids and the like) is eliminated. The DAS existing in the water phase is obtained as osazon together with that formed by oxidation after reaction with 2,4-dinitrophenylhydrazine.

For determination in cancer tissue (3 g) and liver (5 g) one animal was enough for one reading. In the case of testicles, spleen, brain and kidneys tissue from many (usually 3) animals was taken together. From this results the smaller number of single values for these organs, as Table 1 shows.

Table 1. (Continued)

Yoshida-Sarkom (vorgeschritten)										
	33,7	39,3	18,7	19,9	9,3	26,4	28,0	29,5	22,5	29,5
	34,9	48,7	16,2	16,2		26,9	24,0	25,9	18,9	28,5
	40,0	48,7	17,4	17,7		25,4	25,9	26,4	13,2	28,5
	28,6	33,7	15,9	16,4						
	30,0	36,8	20,3	20,4						
	30,1	36,8	14,3	19,5						
	20,4	25,9	16,4	20,8						
	33,7	53,3	15,7	18,9						
	48,8	55,4	17,1	20,5						
Mittel:	33,4	42,1	16,9	18,9		26,2	26,0	27,3	18,2	28,8
		± 9,91		± 1,72						
Yoshida-Sarkom (Anfangsstadium)										
	26,5	28,5	17,1	19,2	17,1	29,5	24,0	29,0	19,2	33,1
	25,9	29,0	16,0	18,9	21,3	31,6	25,2	27,4	20,7	26,9
	23,1	30,5	17,5	18,6						
	25,9	31,6	17,9	20,5						
	36,1	41,4	21,2	27,9						
	25,9	30,0	17,7	19,2						
			17,0	17,6						
Mittel:	27,2	31,8	17,8	20,3	19,2	30,5	24,6	28,2	20,0	30,0
		± 4,81		± 3,47						
ES 4-Sarkom (vorgeschritten)										
	32,5	33,4	17,0	17,3	20,9	25,9	20,2	20,4	18,1	19,1
	32,5	38,6	17,2	18,2	22,4	26,3	18,1	18,8	17,4	24,8
	32,4	36,4	12,9	15,8	25,3	38,8	24,6	25,4	20,4	26,4
	26,8	37,8	19,5	19,8	31,3	37,8	27,1	34,7		
	34,9	38,8	17,2	17,6						
	34,3	41,9	15,0	17,0						
	32,2	40,9	12,2	14,2						
	35,8	48,7								
	37,0	53,8								
Mittel:	33,1	41,1	15,9	17,2	25,0	32,2	22,5	24,8	18,6	23,4
		± 6,36		± 1,77						
ES 4-Sarkom (Anfangsstadium)										
	18,3	21,7	14,4	17,4	8,9	26,7	21,1	22,2	15,1	20,9
	30,4	36,8	13,5	16,4	6,8	24,2	21,0	23,7	13,4	24,7
	15,6	21,2	15,5	16,1			12,7	20,0		
	27,4	30,5	14,3	15,8						
	22,1	22,8	15,1	15,8						
	22,8	24,8	15,2	16,1						
			9,4	13,6						
Mittel:	22,8	26,3	13,9	14,1	7,9	25,5	18,3	22,0	14,2	22,8
		± 5,84		± 2,24						
Durchschnittswerte aller Tumortiere										
	28,5	35,2	15,5	18,0	17,5	30,1	21,5	25,2	10,9	27,5
		± 8,51		± 2,76		± 9,32		± 5,03		± 6,07

Table 1. (Continued)

Yoshida-Sarkom (vorgeschritten)										
	33,7	39,3	18,7	19,9	9,3	26,4	28,0	29,5	22,5	29,5
	34,9	48,7	16,2	16,2		26,9	24,0	25,9	18,9	28,5
	40,0	48,7	17,4	17,7		25,4	25,9	26,4	13,2	28,5
	28,6	33,7	15,9	16,4						
	30,0	36,8	20,3	20,4						
	30,1	36,8	14,3	19,5						
	20,4	25,9	16,4	20,8						
	33,7	53,3	15,7	18,9						
	48,8	55,4	17,1	20,5						
Mittel:	33,4	42,1	16,9	18,9		26,2	26,0	27,3	18,2	28,8
		± 9,91		± 1,72						
Yoshida-Sarkom (Anfangsstadium)										
	26,5	28,5	17,1	19,2	17,1	29,5	24,0	29,0	19,2	33,1
	25,9	29,0	16,0	18,9	21,3	31,6	25,2	27,4	20,7	26,9
	23,1	30,5	17,5	18,6						
	25,9	31,6	17,9	20,5						
	36,1	41,4	21,2	27,9						
	25,9	30,0	17,7	19,2						
			17,0	17,6						
Mittel:	27,2	31,8	17,8	20,3	19,2	30,5	24,6	28,2	20,0	30,0
		± 4,81		± 3,47						
ES 4-Sarkom (vorgeschritten)										
	32,5	33,4	17,0	17,3	20,9	25,9	20,2	20,4	18,1	19,1
	32,5	38,6	17,2	18,2	22,4	26,3	18,1	18,8	17,4	24,8
	32,4	36,4	12,9	15,8	25,3	38,8	24,6	25,4	20,4	26,4
	26,8	37,8	19,5	19,8	31,3	37,8	27,1	34,7		
	34,9	38,8	17,2	17,6						
	34,3	41,9	15,0	17,0						
	32,2	40,9	12,2	14,2						
	35,8	48,7								
	37,0	53,8								
Mittel:	33,1	41,1	15,9	17,2	25,0	32,2	22,5	24,8	18,6	23,4
		± 6,36		± 1,77						
ES 4-Sarkom (Anfangsstadium)										
	18,3	21,7	14,4	17,4	8,9	26,7	21,1	22,2	15,1	20,9
	30,4	36,8	13,5	16,4	6,8	24,2	21,0	23,7	13,4	24,7
	15,6	21,2	15,5	16,1			12,7	20,0		
	27,4	30,5	14,3	15,8						
	22,1	22,8	15,1	15,8						
	22,8	24,8	15,2	16,1						
			9,4	13,6						
Mittel:	22,8	26,3	13,9	14,1	7,9	25,5	18,3	22,0	14,2	22,8
		± 5,84		± 2,24						
Durchschnittswerte aller Tumortiere										
	28,5	35,2	15,5	18,0	17,5	30,1	21,5	25,2	10,9	27,5
		± 8,51		± 2,76		± 9,32		± 5,03		± 6,07

Table 1. (Continued)

Yoshida-Sarkom (vorgeschritten)										
	33,7	39,3	18,7	19,9	9,3	26,4	28,0	29,5	22,5	29,5
	34,9	48,7	16,2	16,2		26,9	24,0	25,9	18,9	28,5
	40,0	48,7	17,4	17,7		25,4	25,9	26,4	13,2	28,5
	28,6	33,7	15,9	16,4						
	30,0	36,8	20,3	20,4						
	30,1	36,8	14,3	19,5						
	20,4	25,9	16,4	20,8						
	33,7	53,3	15,7	18,9						
	48,8	55,4	17,1	20,5						
Mittel:	33,4	42,1	16,9	18,9		26,2	26,0	27,3	18,2	28,8
		± 9,91		± 1,72						
Yoshida-Sarkom (Anfangsstadium)										
	26,5	28,5	17,1	19,2	17,1	29,5	24,0	29,0	19,2	33,1
	25,9	29,0	16,0	18,9	21,3	31,6	25,2	27,4	20,7	26,9
	23,1	30,5	17,5	18,6						
	25,9	31,6	17,9	20,5						
	36,1	41,4	21,2	27,9						
	25,9	30,0	17,7	19,2						
			17,0	17,6						
Mittel:	27,2	31,8	17,8	20,3	19,2	30,5	24,6	28,2	20,0	30,0
		± 4,81		± 3,47						
ES 4-Sarkom (vorgeschritten)										
	32,5	33,4	17,0	17,3	20,9	25,9	20,2	20,4	18,1	19,1
	32,5	38,6	17,2	18,2	22,4	26,3	18,1	18,8	17,4	24,8
	32,4	36,4	12,9	15,8	25,3	38,8	24,6	25,4	20,4	26,4
	26,8	37,8	19,5	19,8	31,3	37,8	27,1	34,7		
	34,9	38,8	17,2	17,6						
	34,3	41,9	15,0	17,0						
	32,2	40,9	12,2	14,2						
	35,8	48,7								
	37,0	53,8								
Mittel:	33,1	41,1	15,9	17,2	25,0	32,2	22,5	24,8	18,6	23,4
		± 6,36		± 1,77						
ES 4-Sarkom (Anfangsstadium)										
	18,3	21,7	14,4	17,4	8,9	26,7	21,1	22,2	15,1	20,9
	30,4	36,8	13,5	16,4	6,8	24,2	21,0	23,7	13,4	24,7
	15,6	21,2	15,5	16,1			12,7	20,0		
	27,4	30,5	14,3	15,8						
	22,1	22,8	15,1	15,8						
	22,8	24,8	15,2	16,1						
			9,4	13,6						
Mittel:	22,8	26,3	13,9	14,1	7,9	25,5	18,3	22,0	14,2	22,8
		± 5,84		± 2,24						
Durchschnittswerte aller Tumortiere										
	28,5	35,2	15,5	18,0	17,5	30,1	21,5	25,2	10,9	27,5
		± 8,51		± 2,76		± 9,32		± 5,03		± 6,07

Table 1 shows

a) a significantly increased total vitamin C content in tumor tissue as compared with normal tissue [liver,* brain,* testicles* and spleen ($p < 5\%$)] of the same animals,

b) a decline of the values of total vitamin C in the organ tissues (liver,* spleen, brain) of tumor rats as compared with normal rats,

c) an increased concentration of vitamin C in tumors with rapidly developing growths in ES 4-sarcoma and Yoshida-sarcoma ($p < 5\%$),

d) a significantly higher vitamin content in the virulent Yoshida-sarcoma, Walker-carcinosarcoma 256 and Jensen-sarcoma tumors as compared with the less rapidly growing Chlorom[1]),

e) the existence of DAS (difference between total vitamin C and L-ascorbic acid) in all tissues examined, including the tumors. The values fluctuate very considerably; the differences narrow essentially in most cases however if one does not consider DAS in isolation but in the framework of total vitamin C.

Some comparable determinations of the situation with respect to tumor-bearing guinea pigs are summarized in Table 2.

From Table 2 it appears that the total vitamin C content in the tumor is higher than in the normal organs of the tumor animals chosen for comparison, including the liver ($p < 5\%$). The concentration of total vitamin C in the liver of the tumor-bearing guinea pigs was lowered in comparison with the normal liver ($p < 5\%$).

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[1] Statistically verified ($p < 1\%$).

The organ and tumor tissue whose total vitamin C content is summarized in Table 2 was also examined with 2,6-dichlorophenolindophenol for the quantity of AS contained therein. We refused to insert these values in Table 2 because ~~because~~ some of these determinations gave a lower value for total vitamin C than for AS alone. The mistake must be sought in the differing specifications of the two indicators used. Only the values for total vitamin C are therefore to be considered real. After transformation of total vitamin C into the dehyroformupon determination with 2,4-dinitrophenylhydrazine the participation of reacting substances is plainly diminished, and indeed into the same substances which no reaction-capable Ketone-groups seek out; 2,4-dinitrophenylhydrazine reacts only with ketone-groups with the corresponding hydrazon. The added AS - as is shown in frequently repeated injection tests in rat and guinea pig liver - reappears in practice in quantitative amounts, if the addition is at the same time followed with oxalic acid, the conclusion must be drawn that in guinea pig organs reducing substances are present which are quickly oxidized by 2,6-dichlorophenolindophenol like AS and thereby deceptively show higher values for AS. In this case the basic principle underlying Feldheim's method is denied, of eliminating the reactants on the basis of different rates of oxidation with Tillman's reagent (for long the standard reagent for determination of AS). The probability that these sources of error play a still bigger role for the values given by other authors for guinea pig organs- since with the Feldheim modification at least the slower reacting reactants can be eliminated - might offer among other things a possibility of clarification for the often strongly diverging findings in the literature.

Table 2. Total Vitamin C Content (mg/100 g Wet Weight) of Daels Guinea Pig Sarcomas and Organs of Tumor-bearing and Normal Guinea Pigs.

	Tumor	Leber	Hirn	Niere
Tumortiere				
	19,4	16,7	15,4	9,6
	20,4	14,5	16,7	
	22,3	13,3		
	17,0	10,5		
	15,5			
Mittel:	18,9 ± 2,74	13,8 ± 2,58		
Normale Kontrolltiere				
		11,4	11,2	9,0
		22,0	14,2	11,1
		20,7		
		26,9		
		17,6		
Mittel:		19,7 ± 5,73		

(Tumertiere= Tumor animals; Tumor=Tumorm; Lebr=Liver; Hirn=Brain; Niere=Kidneys; Mittel= Average; Normale Kontrolltiere= Normal Control Animals; Mittel= Average).

Whether these methodical sources of error possibly play a role, even though doubtfully smaller, in rat organs, cannot now be excluded with full certainty.

Discussion

Preceding Findings on the vitamin C content of tumors and tumor-bearers

We could find no information in the literature on the DAS content of tumors. In contrast there are many findings for AS, which indeed are often contradictory. Low values in more or less clear formulations (47,161,164,178,146) stand in contrast to accounts that no clear difference distinguishes (117,175,189), or record increased values, at least with respect to the tumor. A part of the work is limited only to the tumor tissue or only to organs of tumor-bearers (29,131,177,193,194), which naturally evaluation restricts, especially differences of experimental technique and the already mentioned methodological sources of error, any comparison of numerical values of different investigators only with the great limitations noted. Other authors test

the vitamin C content of tumors in comparison with the normal tissue of tumor-bearers, without bringing in for comparison also normal control animals. Further, almost all previous publications are based on too small a number of animals to make a statistical evaluation possible. It would take too much space to discuss in detail the multitude of previous, for the most part older publications (6, 18, 19, 28, 35, 36, 51, 56, 61, 84, 92, 94, 118, 121, 125, 158, 185, 188, 189, 190, 196).

Considered altogether, the earlier works then show that the concentration of vitamin C in the tumor is absolutely and relatively higher than the content of other vitamins, which for the most part are strongly diminished.

The weight of the works that gave higher values ought, on the ground of breadth of materials evaluated, to be rated higher than the eralier-mentioned reports. Thus in an investigateion by W.V.B. Robertson (147) the vitamin C content of 22 tumors (mouse and rat) amounted to 15 to 70 mg per 100 g fresh weight. In hepatoma he placed 31 of the rats on an average 32 mg higher than in normal, regenerating or fetal liver (maximum 21 mg %). Also in 2 other rat tumors there was more vitamin C contained than in normal organs (liver, spleen, kidneys), apart from the adrenals. (189).

According to M.H. Briggs (31) the vitamin C content in butter-yellow hepatomas according to determination by another method was at 25 μg/g likewise higher than in normal liver (20 μ g/g). Feeding with nitrogenous dyestuff and 2-acetylaminofluorine increased the AS-content in mouse and rat liver (41), as did injection of carcinogen sybstance (48, 94).

Carcinogen substances appear generally to accelerate the synthesis and excretion of vitamin C (30, 35, 44, 93, 191).

Only relatively few investigations exist relating to the vitamin C content in human tumors. Most of the researchers limited themselves to determination of vitamin C deficits by AS-determinations in the urine of cancer sufferers after intake of vitamin C.

The vitamin C concentration in human tumors shows strong fluctuations according to one investigation (70) from traces to 4.5 mg%, for the greatest part in dependence on intake of exogenous vitamin C. That is only about one-tenth the value for tumors in vitamin C-synthesizing animals. In a liver carcinoma of human origin there was only 0.04 mg/g of vitamin C (131).

A new Japanese publication is available on the AS-content of human cervical carcinoma (174). In general among humans the vitamin C amount is also higher in the tumor than in the normal excretory tissues (198).

According to an older study (1936) on tumor-bearing guinea pigs virulent sarcoma tissue contained 0.4 mg/g of AS, necrotic tissue in contrast almost none (29).

Physiological Significance of Vitamin C

Vitamin C serves as a hydrogen-carrier in various reaction chains of metabolism, especially in the decomposition of carbohydrates, in the oxidation of fatty acids and in amino acid metabolism. As an oxidation-reduction system it is thereby divided into different fermentation systems. In a certain sense it displays itself then as an activator of cell metabolism.

1. Carbohydrate Metabolism

A great number of individual observations exist here (see 76, 142), which however still permit no exhaustive survey. Some of them are: In experimental scurvy the blood sugar is increased, sugar-tolerance, liver phosphorylasis and insulin content of the pancreas (β-cell degeneration) are lowered and liver glycogen diminished (13, 15, 130). With simultaneous intake of glucose and AS, the storage of glycogen in the liver is larger than with the supply of glucose alone (157). Prolonged insulin treatment leads to improvement of sugar tolerance and glycogen synthesis (14). Ample vitamin intake increases the effect of adrenal hormone on glycogen synthesis and storage (11).

2. Amino Acid and Nucleic Acid Metabolism

The decomposition of aromatic amino acids, especially of tyrosine and phenylalanine, in the liver is dependent on the presence of vitamin C (105, 109, 163). The effect of AS on Tyrosine and phenylalanine oxidation appears to have the character of a coenzyme (49, 75, 78, 135). A whole list of findings (62, 64, 152) speaks likewise for the participation of vitamin C in nucleic acid metabolism. The albumin and RNA content of the liver in scorbutic guinea pigs amounts to less than half the normal values (57). The RNA-DNA ratio of spleen and adrenals in guinea pigs is likewise shifted (63) after feeding with a scurvy-producing diet .

3. The Participation of AS in the Biosynthesis of Steroid Hormones

Vitamin C is found in the adrenals in the highest concentration of all organs. One assumes that in the oxidation of AS to monohydroascorbic acid in the cortex of the adrenals hydroxyl radicals are released which are carried to the steroid molecule by hydroxylasis (95, 178). The hydroxylation function appears to have general significance, for example

also in the transformation of proline and tryphophan into hydroxyproline and 5-hydroxytryptophan under the influence of AS (39, 40, 149). Vitamin C is required for certain oxidations in steroid hormones, for example in deoxycorticosteron (91, 165), likewise for oxidation of fatty acids, for example of linolic acid in the brain (25,46). However the last does not appear to be valid for other organs. Further, a catalytic function is attributed to vitamin C in the oxidation of phospholipoids in the rat liver (153).

The effects of stress, which leads to an increased output of adrenal hormones, for example operations, fever, etc., lead to a strong rise of vitamin C consumption. Also the requirement for AS is increased in irradiation, wherein it remains open whether vitamin C participates as a reducing means for neutralization of certain toxic irradiation products, e.g, peroxide, or only indirectly through an activation of RES [RNA?]. In any case radiation tolerance rose after doses of vitamin C and A.

4. Relations to other vitamins

AS participates in the transformation of folic acid into folin [?] acid (74, 86, 103, 114, 133). Since folic acid also is active in tyrosine metabolism the possibility exists that the effect on tyrosine metabolism is achieved indirectly through the folic acid.

Certain relations exist between Vitamin C and A. A deficiency of A leads to a decrease of AS reserves in various organs (89, 97, 116, 176), which even in animals able to sythesize their own AS can take such dimensions that scurvy-like symptoms appear (115). Should the case be reversed, with a deficiency of AS vitamin A excretion is pathologically increased, whereby a secondary deficiency of vitamin

A can occur.(161). Toxic doses of vitamin A on the other hand bring about an abnormal lowering of vitamin A C content in the adrenals (37, 151).

Vitamin B_{12} prevents too rapid an oxidation of AS to DAS (129).

5. Effect on Iron Metabolism

Hypochrome scurvy anemia is brought about by a disturbance of iron metabolism, which can be diminished by AS (2, 12, 65, 71, 181).

6. Influence on Mucopolysaccharid Metabolism

In the metabolism of the basic substance and collagen fibers vitamin C plays the leading role.(67, 87, 41, 167, 192). The biosynthesis of collagen rests on the transfer of the proline in pre-collagen to hydroxyproline (16, 33, 66, 68, 149, 150). Also the synthesis of hyaluron acid, nemessary for the building up of the intracellular cementing substance, is vitamin C-dependent. The petechial [?] hemorrhages appearing in scurvy are related to it.

7. Resorption, Decomposition and Excretion

Mechanism of the effect

Vitamin C uptake is achieved through the small intestine (104, 137). In experiments on the burden in humans there were excreted as decomposition products, besides AS, diketoglon acid and oxalic acid; 5 % of the applied AS ($1\text{-}C^{14}$) was detected as respiratory CO_2. In guinea pigs one finds over 60 % of the introduced dose in exhaled CO_2 (32, 81).

As the specifically active form of AS monodehydroascorbic acid, a radical intermediate product of oxidation of AS to DAS, has recently been assumed (101), which is re-formed to AS by DPNH - II monodehydroascorbic acid - transhydrogenase. The oxidation of AS to monodehydroascorbic acid is apparently catalyzed by zytochrome b_5, wherein it is not yet decided whether this reacts directly with acid materials (95, 101).

8. Findings in Cancer

Vitamin C is found in increased concentrations in the most important endocrine glands, in young rapidly growing tissues and also in wound regeneration; there likewise exists a direct connection between the functional activity of the corpus luteum and vitamin C content. It will therefore be expected that the significance of vitamin C in cancer should be considered in connection with the increased metabolism of the cancer cell (124); AS functions in the cancer cell as as "breathing ferment".

The daily vitamin C consumption of patients with advanced cancer amounts - after satisfaction of deficits- is about double that of control cases, with 125 mg as compared with an average of 67 mg.(108).

A strong vitamin C deficit, especially in later stages, has been confirmed by numerous investigators (3 to 5, 27, 43, 58, 69, 73, 88, 100, 121, 160 to 162, 171, 184). G. Gaethgens (58) for instance in 20 gynecological cancer cases within a few days reported up to 6000 mg (500 mg daily) wihout a rise of the reduction value of the urine. In many cancer cases the deficit could not be exactly ascertained at all. W. G. Deucher (43) recorded vitamin C deficits in cancer patients of between 2500 and 11000 mg (average 4300 mg), E. Schneider (160) an average of 4550 mg as compared with 1350 mg in other illnesses, and so forth. Remarkably, one is careful to find no symptoms of scurvy in spite of these high deficits in cancer patients, although the values given in part far exceed the deficit in scurvy. Gaethgens seeks to explain the vitamin C deficit by an increased oxidative destruction of vitamin C in cancer patients' organism.

The tumor has a higher affinity for vitamin C than the normal tissues

investgiated by us; that is because the enrichment of the tumor is acheived at least partly at the cost of the organs (see Table 1). The increased storgae capacity in the tumor might be primarily responsible for the vitamin C deficit, that is, it is probably in great part a question of a vitamin C deficit of the growth itself. Only thus can the absence of scorbutic phenomena be satisfactorily explained in view of the height of the AS quantities necessary for staisfaction: the increased affinity of the tumor for vitamin C plainly does not go so far that the vitamin C shifts within the tumor-bearing organism lead to a complete de-vitaminization of other organs. Schneider tries to explain the absence of scurvy symptoms by assuming that the vitamin C introduced is not used directly to cover the AS deficit, but primarily for ~~synthe~~ synthesis of glycogen.

As in cancer patients, in guinea pigs too the vitamin C reserves are quickly exhausted by a growing tumor (193).

It is not to be assumed that the organism supplies the tumor with vitamin C according to its strength in order to bring the tumor growth to a halt, somehwat as antibodies are transported to the site of defensive action; the quickly growing tumor probably covers its vitamin C requirement much as the muscular tissue melts away to satisfy its energy andand building materials requirement. One must keep these data in mind also in therapeutic consideration with vitamin C. As is known, oxidation is incomplete in the tumor because of the relatively low respiration in comparison with the increased glycolysis (aerobic glycolysis). Even if one therefore wished to increase the respiration by an addition of vitamin C one would apprently only open to the tumor a new source of energy, which could scarcely exercise a carcinostatic effect. Increases of respiration in vitro - in tumor cells- have been reported by DAS (80) as well as

the increase of O_2 consumption in ~~normalzandzscorbutic~~ liver samples of normal and scorbutic guinea pigs by AS (173). This increase however in the case of the liver was equivalent to AS dose, which permits the conclusion that it was caused by oxidation of the added vitamin C itself and not by a genuine increase of respiration. With some reserve one must first judge the reduction of anerobic glycolysis of tumorm samples in vitro at about 34 to 68 % in dependence on the added quantities of DAS, because unphysiologically high doses were used. Besides, these results stand in a certain contradiction with the therapeutic experiments of the smae authors on rat tumors: here, the case being reversed, just by high DAS doses no effect was obtained, but only by comparatively small quantities.

DNA synthesis in a virulent rat sarcoma was promoted in vitro by addition of AS - in contrast to normal rat liver- (62, 63). In agreement therwith the DNA content in a guinea pig sarcoma was diminished by the feeding of scorbutogenic food. (168).

A plausible interpretation of a possible limiting effect of vitamin C addition on tumor growth, with immediate attack point in the cancer cell itself, under these circumstances for long could nto be be given. Where favoravorable experiences were undergone by cancer patients or tumor-bearing animals it might be a question of indirect effects, of an increase of defensive strength through an improvement of of the general condition after removal of the vitamin C deficit. Whether AS or DAS was used might not be decisive, since in the liver a rapid regeneration of DAS to AS occurs (140). Especially before and afeer operations and in X-ray irradiation, which itself causes an increase in vitamin C consumption, a removal of the vitamin C deficit might be recommended (see also K. H. Bauer (20)). On the other hand

an overdose of vitamin A in combination with AS treatment should, in view of the mentioned findings, be avoided. Unfortunately it cannot be denied that subjective considerations in the judgment of clinical consequences are in many cases not without influence. For the evaluation of the development of many cancer forms, especially in internal organs, clear criteria are often lacking; fully similar human cancer cases which might serve as controls without additional handling are at our disposal, besides, only in exceptional cases. Thus there is no lack of differing ideas. E. Schneider (160 to 162) and v. Wendt (195) saw favorable effects throguh high doses of vitamin A and C (2000 mg daily) with limitation of cancer growth and increased tolerance of X-ray irradiation. On the other hand, a better response was observed in Lg-patients who were kept on a vitamin C-free diet during several weeks of irradiation than in control cases (123).

Thus a vitamin C-free diet was prescribed for this purpose (122).

L. Huber (83) recorded in 25 grave gynecological cancer cases, which were treated with same doses as those treat by v. Wendt an improvement in general condition but no influence on the tumors.

Outstanding improvement of general conditon and partial regressive pehnomena were further reported by H. Reinhold (145) by increased additions of vitamin A and C in 218 mostly inoperable cancer patients. Other authors (7, 8, 22, 23, 98, 126, 128, 143, 156, 179, 187) report more or less favorable effects at least on the general conditon by AS alone or in combination with A, in part also on the blood picture in anemia, leukemia and irradiation (1, 21, 26, 34, 38, 50, 59, 72, 79, 85, 110, 132, 139, 189).

Favorable influence on the course of the illness in cancer by DAS was also reported (9, 24, 111, 112, 155). In experimental tumors

Substantially larger is the number of works that report a more or less deliberate stimulation of tumor growth by vitamin C (45, 54, 55, 96, 107, 113, 197). Unfortunately no statistical evaluation exists for the greatest part of the earlier animal exprimental works that report a limiting or accelerating effect of vitamin C enrichment, especially for the largest part of those experiments carried out on animals that can synthesize vitamin C themselves (rat and mouse).

The great majority of investigators could confirm no clear influence on the development of animal transplantation tumors (10, 17, 42, 82, 99, 106, 120, 127, 134, 136, 138, 144, 172, 198). The fact alone that the clinical course of ~~virulent~~ malignant tumors does not differ essentially in animals with and without the capacity to synthesize vitamin C speaks against ~~the~~ a decisive significance either of AS or DAS in tumor growth.

On a scorbutic diet a transplantable fibrosarcoma in guinea pigs grew more slowly than on a normal diet, wherein the vitamin C content in the tumor fell almost to nil (148, 169). In this connection it should be kept in mind that in animals in reduced general condition transplantation tumors generally grow more poorly.

A series of further experimental works should at least be mentioned (77, 90, 119, 154, 166, 180, 183, 186).

In 5 small test series (see Table 3) in which guinea pigs received high vitamin C doses after transplantation of the Daels-sarcoma, a tendency was recognizable for better tumor growth in animals treated with AS.

Table 3. Influence of Increased Vitamin C Intake (50 mg daily) on Occurrence of Daels-sarcoma in Guniea Pigs

	Kontrolltiere ohne Zusatzbehandlung mit Vitamin C		mit Vitamin C behandelte Versuchstiere	
	Tierzahl	Angangsziffer	Tierzahl	Angangsziffer
	7	1	4	—
	4	4	4	1
	9	4	10	3
	12	2	6	1
	5	—	4	—
Summe:	37	11 (30%)	28	5 (18%)

(1st section, cols 1 & 2, Control animals without Supplemental treatment with vitamin C; cols. 3 & 4, Experimental Animals treated with vitamin C)

(2nd section, Col. 1, no. of animals; Col. 2, no. of occurences; Col. 3, no. of animals; col. 4 nol of occurrences)

(3rd section, Sum)

Summary

In investigations of 64 tumor-bearing and normal rats (5 different transplantation tumors) we found:

1. The total vitamin C content of tumor tissue was higher than in the parenchymatose organs of the tumor-rats which were examined [liver*, brain*, testicles*, and spleen ($p < 5\%$)].

2. The concentration of total vitamin C in the liver*, spleen and brain of tumor animals was lower than in the corresponding tissues of normal rats. The enrichment in the tumor was therefore at least partially at the cost of the vitamin supply to other organs.

3. The values obtained for total vitamin C in the virulent Yoshida-sarcoma, Walker-carcinosarcoma 256 and Jensen-sarcoma tumors was higher

than in the slower growing Chlorom*.

4. Vitamin C content was increased in ES 4-sarcoma* and Yoshida-sarcoma ($p < 5\%$) with excessive tumor development

5. In guinea pigs the vitamin C concentration is dependent on food conditions. Reducing reactant substances play here a bigger role than in rat organs upon use of 2,6-dichlorophenolindophenol. With 2,4-dinitrophenylhydrazine as the indicator tumor tissues in guinea pigs also show higher total vitamin C values than liver ($p < 5\%$), kidneys and brain; likewise the values in tumor animal liver are lower than in normal liver ($p < 5\%$).

6. DAS was detectable in various amounts in all organ tissues investigated, including tumors.

7. The limitations of the methodology used are pointed out. The values found for DAS are minimal, those for AS in contrast are maximal values.

8. The numerical occurrence and growth of Daels guinea pig sarcoma was not significantly influenced by hypervitaminization with vitamin C; there existed rather a tendency to growth promotion than to limitation of the tumor.

9. A survey of earlier findings on the relations between vitamin C and tumors in man and animals is given.

*The conclusions marked * have been statistically verified ($p < 1\%$).
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V. AUS DEM AUSLAND

Aus der II. Medizinischen Klinik der Medizinischen Akademie Lublin (Professor Dr. A. R. Tuszkiewicz)

Ferienexpedition der Medizinischen Akademie und des Institutes für Arbeitsmedizin und Hygiene auf dem Lande in Lublin

Allgemeine Charakteristik, Aufgaben und Organisation

Von Alfred R. Tuszkiewicz

Vom Jahre 1954 an organisieren die Medizinische Akademie und das Institut für Arbeitsmedizin und Hygiene auf dem Lande in Lublin während der Sommerferien (Juli oder August) sog. sozialwissenschaftliche Ferienexpeditionen in verschiedenen Dörfern der Wojewodschaft Lublin. Die Initiative der Organisation dieser Ferienexpeditionen ging vom Ministerium für Gesundheitswesen aus. Das Ziel dieser Ferienexpeditionen war vor allem, das Land den Studenten näherzubringen. Die Studenten sollten nicht nur den Gesundheitszustand der Landbevölkerung, sondern auch im innigen Kontakt ihr Leben und ihre Arbeit näher kennenlernen. Gleichzeitig sollten die Ferienexpeditionen der Landbevölkerung der in ihrer Entwicklung zurückgebliebenen Regionen Polens die Errungenschaften des sozialistischen Gesundheitswesens vor die Augen führen und seine Anteilnahme an der Besserung des Gesundheitszustandes der Landbevölkerung veranschaulichen. Eine weitere Aufgabe der Ferienexpeditionen war, die hygienische Aufklärung und die allgemein kulturelle und politische Tätigkeit zu entwickeln. Die Ferienexpeditionen realisierten nicht nur diese Ziele, sondern entwickelten auch eine rege Tätigkeit auf dem ärztlich-therapeutischem und auf dem wissenschaftlichem Gebiet (s. unten).

Die erste der Ferienexpeditionen, die im Jahre 1954 in einem staatlichen Gut organisiert wurde, hatte nur 14 Teilnehmer und arbeitete ohne einen systematischen Plan. Ihre Erfahrungen dienten aber als Grundlage für die systematische Planung späterer Expeditionen. An allen weiteren Expeditionen nahmen 70—80 Personen teil, davon durchschnittlich 20 Assistenten der Medizinischen Akademie und des Institutes, 40 Studenten des 3. und 4. Jahrgangs der Medizinischen Akademie sowie 15 technische Assistenten und Arbeiter der wirtschaftlichen und Administrativen Abteilung der Medizinischen Akademie. Folgende Kliniken und Anstalten beteiligten sich an den Eypeditionen:

II. Medizinische, I. Chirurgische, I. Gynäkologische, Pädiatrische, Orthopädische, Laryngologische, Stomatologische, Dermatologische, Psychiatrische, Radiologische und Ophthalmologische Klinik der Medizinischen Akademie sowie die Klinische Abteilung, Anstalt für Hygiene, Anstalt für Parasitologie und Anstalt für Ernährung des Institutes. Leiter der ersten Ferienexpedition war Prof. Dr. Jan Danielski, Leiter des Katheders für Hygiene der Medizinischen Akademie und gleichzeitig Leiter der Anstalt für Landhygiene des Institutes, welcher große Verdienste an der Organisation der Ferienexpeditionen hatte. Nach seinem Tode ging die Leitung der Ferienexpeditionen in die Hände der Dozenten Dr. W. Szewczykowski über, der Mitarbeiter von Prof. Dr. A. Tuskiewicz in der II. Medizinischen Klinik der Medizinischen Akademie und gleichzeitig in der Klinischen Abteilung des Institutes war. Während die Arbeit des klinischen Sektors der Ferienexpeditionen vom Dozenten Dr. Szewczykowski persönlich geleitet wurde, wurden die hygienischen Untersuchungen von Dr. Br. Wawrzyszuk geplant und geführt, Oberarzt der Anstalt für Hygiene des Institutes und ältester Mitarbeiter von Prof. Dr. Danielski.

Die Assistenten waren von den Leitern der Kliniken bzw. Anstalten auserwählt, die Studenten meldeten sich freiwillig.

Die Ferienexpeditionen arbeiteten einen Monat lang in einem auserwählten Dorf der Wojewodschaft Lublin, jedes Jahr in einem anderen, wobei eine schöne Lage der Ortschaften und die Möglichkeit guter Erholung für die Studenten (Wald, Wasser) immer bei der Wahl mit in Betracht gezogen wurde. Die 9 Gemeinden, in welchen die Expeditionen in den Jahren 1954 bis 1962 lokalisiert wurden, zeigten verschiedene soziale, ökonomische und kulturelle Verhältnisse. 2 waren abgelegene, arme Dörfer, ohne Landambulatorium (nächster Arzt 10 km entfernt), 2 Siedlungen mit Industrieanlagen (Obstverarbei-

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IV. THEORETISCHE UND EXPERIMENTELLE MEDIZIN

Aus der Forschungsstelle für Experimentelle Onkologie (Leiter: Dr. med. habil. F. Schmidt)
und dem Institut für Ernährung (Präsident: Professor Dr. Dr. Dr. K. Lohmann)
der Deutschen Akademie der Wissenschaften zu Berlin in Potsdam-Rehbrücke

Vitamin C und Krebs

Von **F. Schmidt, H. Schmandke** und **B. Gassmann**

Das Vitamin C hat bisher von allen Vitaminen im Zusammenhang mit malignen Tumoren das größte Interesse gefunden. Ein Grund dafür mag darin zu suchen sein, daß dieses Vitamin vor allen anderen im Tumorgewebe mengenmäßig absolut und relativ in der höchsten Konzentration vorkommt. Eine Reihe von Veröffentlichungen gilt ferner auch gewissen Therapieerfolgen bei malignen Geschwülsten mit Vitamin C. Das Studium der Literatur ergibt jedoch so viele Widersprüche und abweichende Befunde, daß wir es für angebracht hielten, einige Teilfragen zu diesem Thema einer erneuten experimentellen Prüfung zu unterziehen.

Wir untersuchten insbesondere den Gehalt verschiedener Rattentransplantationstumoren an L-Askorbinsäure (AS) und Dehydroaskorbinsäure (DAS) im Vergleich zu parenchymatösen Organen von Tumor- und Normaltieren. Die DAS beanspruchte dabei vor allem deshalb erhöhte Aufmerksamkeit, weil die Monodehydroaskorbinsäure nach neueren Auffassungen (101) als eigentliche Wirkform des Vitamins C gilt und weil die Ansicht vertreten wird, daß DAS in Tumoren nur in äußerst geringen Konzentrationen vorkommt (53, 80). Merkwürdigerweise liegen jedoch gerade zu der letztgenannten Frage noch keine experimentellen Befunde vor, wie eine Sichtung des vorliegenden Schrifttums ergab.

In einigen vergleichenden Untersuchungen prüften wir ferner auch Tumoren und Normalgewebe von Meerschweinchen auf den Gehalt an AS und DAS, weil hier bessere Vergleichsmöglichkeiten zu den Gegebenheiten beim Menschen vorliegen, da bekanntlich das Meerschweinchen ebenfalls nicht — im Gegensatz zur Ratte — in der Lage ist, Vitamin C selbst zu synthetisieren.

In einigen Testansätzen prüften wir ferner den Einfluß der Zufuhr hoher Dosen an Vitamin C auf Angangsziffer und Wachstum eines transplantablen Meerschweinchensarkoms.

Versuchsmaterial und Methode

Zu den Rattenversuchen wurden Tiere unseres haseigenen Stammes verwendet, die einheitliches Futter eigener Herstellung (159) erhielten.

Ratten mit folgenden Transplantationstumoren wurden untersucht: Walker-Karzinosarkom 256, Jensensarkom, Yoshida-Sarkom, ES 4-Sarkom (60), Chlorom (Shay). Die Tumortiere gehörten verschiedenen Stadien an: Anfangsstadium: Tumor etwa 3 g, vorgeschrittenes Stadium: Tumor etwa hühnereigroß. Das Untersuchungsmaterial wurde von makroskopisch sichtbaren Nekrosen möglichst weitgehend befreit; bei den vorgeschrittenen Tumoren wurden nur die Randpartien verwendet. Insgesamt wurden an 57 Tumorratten außer dem Tumor Leber, Milz, Hoden und Hirn, bei den normalen Kontrollratten auch die Nieren untersucht.

Zu den Testserien an Meerschweinchen wurde das Daels-Sarkom verwendet[1]).

Die therapeutischen Versuche an Meerschweinchen wurden in 5 Serien angesetzt. Dabei erhielten die Tiere der Versuchsgruppe, beginnend 3 Tage vor der intramuskulären Transplantation des Daels-Sarkoms, täglich 50 mg „Ascorvit forte" (VEB Jenapharm) per Schlundsonde oder intramuskulär.

Zur Bestimmung der AS und der DAS wurden die Tumor- und Organgewebe sofort nach der Entnahme in Eiswasser gestellt oder in der Tiefkühltruhe eingefroren, falls die Vitamin-C-Bestimmung nicht sofort angeschlossen werden konnte. Nach der Einwaage folgte eine feine Zerkleinerung mit einem Homogenisator (Ultraturrax, Fa. Janke & Kunkel KG, Staufen i. Br.) in 2%iger Oxalsäure.

Zur Eiweißfällung wurden anschließend 10 m*l* 10%ige Trichloressigsäure zugefügt und auf 50 m*l* mit 2%iger Oxalsäure aufgefüllt. Nach Filtration wurde die in der Probe enthaltene AS nach der Verfahrensweise von W. Feldheim (52) mit überschüssiger 2,6-Dichlorphenolindophenollösung oxydiert. Durch Extraktion des überschüssigen Farbstoffs mit Amylazetat wird die Oxydation nach 15 und 30 Sekunden unterbrochen und die unverbrauchte Oxydationsmittelmenge photometrisch bestimmt. Unter Zugrundelegung der vereinfachten Annahme, daß Störsubstanzen in der Zeit 0 bis 15 Sekunden dieselbe Menge 2,6-Dichlorphenolindophenol verbrauchen wie in der Zeit 15 bis 30 Sekunden, läßt sich der Verbrauch durch Störsubstanzen (Glutathion, Reduktone, Reduktinsäure und dgl.) eliminieren. Die in der wäßrigen Phase vorliegende DAS wird zusammen mit der durch die Oxydation gebildeten nach Reaktion mit 2,4-Dinitrophenylhydrazin als Osazon erfaßt.

Zur Bestimmung im Krebsgewebe (3 g) und Leber (5 g) genügte ein Tier für einen Meßwert. Im Falle von Hoden, Milz, Hirn und Niere wurden die Gewebe von mehreren (in der Regel 3) Tieren zusammengenommen. Daraus resultiert die geringere Anzahl von Einzelmeßwerten bei diesen Organen, wie Tabelle 1 zeigt.

Ergebnisse

Die Ergebnisse der Vitamin-C-Bestimmung bei Normal- und Tumorratten sind in Tabelle 1 zusammengestellt.

[1]) Wir danken Herrn Professor Dr. Thurzo, Onkologisches Institut Bratislava, für die Überlassung dieses Stammes.

Tabelle 1 Gehalt von Transplantationstumoren und Organen von Ratten an Vitamin C [l-Askorbinsäure (1. Zahl) und Gesamtvitamin C (2. Zahl)] in mg% des Feuchtgewichtes

Organe von tumorfreien Normalratten (Kontrolle)												
	Nieren		Leber		Milz		Hoden		Hirn			
	13,7	16,1	24,1	24,4	22,9	28,9	18,9	19,2	27,4	30,0		
	14,2	14,9	22,8	22,9	24,2	33,1	19,7	23,1	24,7	26,2		
	14,9	16,7	24,8	25,6	26,1	34,6	20,4	24,4	25,0	26,7		
	15,2	15,5	25,0	25,0	19,7	27,4	23,8	26,3	31,5	35,7		
	15,9	16,4	21,3	21,7	25,4	28,2	18,7	25,3	30,0	35,1		
			22,8	23,5					29,6	30,4		
			21,0	22,0					30,1	31,4		
Mittel	14,8	15,9	23,1	23,5	23,7	30,5	20,3	23,7	28,3	30,7		
	± 0,72		± 1,94		± 3,20		± 2,76		± 3,69			
Walker-Ca (vorgeschritten)												
	Tumor		Leber		Milz		Hoden		Hirn			
	34,1	34,6	15,7	15,8	20,5	26,7	15,8	20,1	22,7	24,2		
	32,9	35,1	14,8	15,1	21,2	28,9	16,7	20,4	21,5	28,4		
	28,4	32,6	13,7	15,5	11,0	21,6	20,4	25,9	24,3	27,4		
	25,7	26,2	12,5	13,9	18,3	32,1	16,2	29,5	19,5	30,5		
	28,2	32,6	20,2	22,0	13,5	38,8			18,0	27,5		
	22,5	33,7	17,4	21,7	17,7	39,7						
	20,7	34,2	17,8	22,0								
	21,9	33,7	12,6	16,7								
	46,3	49,6	7,1	14,9								
	31,9	35,2										
	31,3	32,6										
	27,1	40,9										
	31,3	37,3										
Mittel:	29,4	35,2	14,6	17,5	17,0	31,3	17,3	24,0	21,2	27,6		
	± 5,41		± 3,37		± 7,05		± 4,54		± 1,99			
Walker-Ca (Anfangsstadium)												
	19,2	35,2	14,0	14,6	12,3	34,2	22,2	27,4	20,1	24,3		
	31,6	46,6	14,1	15,8	12,6	32,6	23,1	28,5	22,5	33,1		
	29,5	40,9	16,9	17,1								
	28,0	37,3	16,5	18,3								
	21,3	32,6	16,0	18,9								
	31,3	35,2	17,6	18,3								
Mittel:	26,8	38,0	15,9	17,2	12,5	33,4	22,7	28,0	21,3	28,7		
	± 5,05		± 1,62									
Jensen-Sarkom (vorgeschritten)												
	32,7	33,1	16,7	17,9	15,9	29,5	18,6	19,2	32,0	35,7		
	29,7	37,3										
Jensen-Sarkom (Anfangsstadium)												
	32,1	33,1	16,3	17,0								
Chlorom (Shay) (vorgeschritten)												
	Tumor		Leber		Milz		Hoden		Hirn			
	10,5	24,3	13,2	20,1	17,7	23,2	25,9	25,9	20,0	29,9		
	11,8	21,7	16,4	24,8								
	21,4	23,7	12,6	21,4								
	20,6	24,7	12,6	16,7								
	22,9	23,2	7,1	14,9								
Mittel:	17,4	23,5	12,4	19,6								
	± 1,19		± 3,90									

Tabelle 1 zeigt

a) einen signifikant erhöhten Gesamt-Vitamin-C-Gehalt im Tumorgewebe gegenüber den untersuchten Normalgeweben [Leber*, Hirn*, Hoden* und Milz ($p < 5\%$)] der gleichen Tiere,

b) eine Senkung der Werte für Gesamtvitamin C in den Organgeweben (Leber*, Milz, Hirn) der Tumorratten gegenüber den Normalratten,

c) eine Erhöhung der Konzentration an Vitamin C im Tumor mit fortschreitender Entwicklung der Ge-

Tabelle 1 (Fortsetzung)

Yoshida-Sarkom (vorgeschritten)										
	33,7	39,3	18,7	19,9	9,3	26,4	28,0	29,5	22,5	29,5
	34,9	48,7	16,2	16,2		26,9	24,0	25,9	18,9	28,5
	40,0	48,7	17,4	17,7		25,4	25,9	26,4	13,2	28,5
	28,6	33,7	15,9	16,4						
	30,0	36,8	20,3	20,4						
	30,1	36,8	14,3	19,5						
	20,4	25,9	16,4	20,8						
	33,7	53,3	15,7	18,9						
	48,8	55,4	17,1	20,5						
Mittel:	33,4	42,1 ± 9,91	16,9	18,9 ± 1,72		26,2	26,0	27,3	18,2	28,8
Yoshida-Sarkom (Anfangsstadium)										
	26,5	28,5	17,1	19,2	17,1	29,5	24,0	29,0	19,2	33,1
	25,9	29,0	16,0	18,9	21,3	31,6	25,2	27,4	20,7	26,9
	23,1	30,5	17,5	18,6						
	25,9	31,6	17,9	20,5						
	36,1	41,4	21,2	27,9						
	25,9	30,0	17,7	19,2						
			17,0	17,6						
Mittel:	27,2	31,8 ± 4,81	17,8	20,3 ± 3,47	19,2	30,5	24,6	28,2	20,0	30,0
ES 4-Sarkom (vorgeschritten)										
	32,5	33,4	17,0	17,3	20,9	25,9	20,2	20,4	18,1	19,1
	32,5	38,6	17,2	18,2	22,4	26,3	18,1	18,8	17,4	24,8
	32,4	36,4	12,9	15,8	25,3	38,8	24,6	25,4	20,4	26,4
	26,8	37,8	19,5	19,8	31,3	37,8	27,1	34,7		
	34,9	38,8	17,2	17,6						
	34,3	41,9	15,0	17,0						
	32,2	40,9	12,2	14,2						
	35,8	48,7								
	37,0	53,8								
Mittel:	33,1	41,1 ± 6,36	15,9	17,2 ± 1,77	25,0	32,2	22,5	24,8	18,6	23,4
ES 4-Sarkom (Anfangsstadium)										
	18,3	21,7	14,4	17,4	8,9	26,7	21,1	22,2	15,1	20,9
	30,4	36,8	13,5	16,4	6,8	24,2	21,0	23,7	13,4	24,7
	15,6	21,2	15,5	16,1			12,7	20,0		
	27,4	30,5	14,3	15,8						
	22,1	22,8	15,1	15,8						
	22,8	24,8	15,2	16,1						
			9,4	13,6						
Mittel:	22,8	26,3 ± 5,84	13,9	14,1 ± 2,24	7,9	25,5	18,3	22,0	14,2	22,8
Durchschnittswerte aller Tumortiere										
	28,5	35,2 ± 8,51	15,5	18,0 ± 2,76	17,5	30,1 ± 9,32	21,5	25,2 ± 5,03	10,9	27,5 ± 6,07

schwülste beim ES4-Sarkom* und beim Yoshida-Sarkom ($p < 5\%$),

d) einen signifikant höheren Gehalt des Vitamins in den virulenten Tumoren Yoshida-Sarkom, Walker-Karzinosarkom 256 und Jensen-Sarkom im Vergleich zu dem weniger wuchskräftigen Chlorom[1]),

e) das Vorhandensein von DAS (Differenz von Gesamtvitamin C und l-Ascorbinsäure) in allen untersuchten Geweben, einschließlich der Tumoren. Die Werte schwanken zwar beträchtlich; die Unterschiede verringern sich in den meisten Fällen jedoch wesentlich, wenn man die DAS nicht isoliert betrachtet, sondern im Rahmen des Gesamtvitamins C.

[1]) Statistisch gesichert ($p < 1\%$).

Einige Vergleichsbestimmungen über die Verhältnisse bei tumortragenden Meerschweinchen sind in Tabelle 2 zusammengestellt.

Aus Tabelle 2 ergibt sich, daß der Gesamt-Vitamin-C-Gehalt im Tumor höher liegt als in den zum Vergleich herangezogenen Normalorganen der Tumortiere, einschließlich der Leber ($p < 5\%$). Die Konzentration an Gesamtvitamin C in der Leber der tumortragenden Meerschweinchen war gegenüber der Normalleber erniedrigt ($p < 5\%$).

Die Organ- und Tumorgewebe, deren Gehalt an Gesamtvitamin C in Tabelle 2 zusammengestellt ist, wurden mit 2,6-Dichlorphenolindophenol auch auf die Menge der darin enthaltenen AS untersucht. Wir haben davon

Tabelle 2 Gehalt des Daels-Meerschweinchensarkoms und der Organe von tumortragenden und normalen Meerschweinchen an Gesamtvitamin C (mg/100 g Feuchtgewicht)

	Tumor	Leber	Hirn	Niere
Tumortiere				
	19,4	16,7	15,4	9,6
	20,4	14,5	16,7	
	22,3	13,3		
	17,0	10,5		
	15,5			
Mittel:	18,9 ± 2,74	13,8 ± 2,58		
Normale Kontrolltiere				
		11,4	11,2	9,0
		22,0	14,2	11,1
		20,7		
		26,9		
		17,6		
Mittel:		19,7 ± 5,73		

abgesehen, diese Zahlenwerte in Tabelle 2 aufzuführen, weil ein Teil dieser Bestimmungen für Gesamtvitamin C niedrigere Werte ergab als für AS allein. Der Fehler muß in der unterschiedlichen Spezifität der beiden verwendeten Indikatoren gesucht werden. Als real sind dabei nur die Werte für Gesamtvitamin C zu betrachten. Nach Umwandlung des gesamten Vitamins C in die Dehydroform bei Bestimmung mit 2,4-Dinitrophenylhydrazin wurde offenbar der Anteil der störenden Substanzen verringert, und zwar um diejenigen Stoffe, die über keine reaktionsfähigen Keto-Gruppen verfügen; 2,4-Dinitrophenylhydrazin reagiert nur mit Keto-Gruppen zu dem entsprechenden Hydrazon. Da zugesetzte AS — wie sich in mehrmals wiederholten Stichproben an Ratten- und Meerschweinchenleber zeigte — praktisch quantitativ wiedergefunden wird, wenn der Zusatz gleichzeitig mit Oxalsäure erfolgt, muß der Schluß gezogen werden, daß in Meerschweinchenorganen reduzierende Substanzen zugegen sind, die durch 2,6-Dichlorphenolindophenol ebenso rasch oxydiert werden wie AS und dadurch erhöhte Werte für AS vortäuschen. In diesem Fall versagt das der Feldheimschen Methode zugrunde liegende Prinzip, die Störsubstanzen auf Grund der unterschiedlichen Oxydationsgeschwindigkeit mit Tillmans Reagenz (das bislang als Standardreagenz für die Bestimmung von AS gilt) zu eliminieren. Die Wahrscheinlichkeit, daß diese Fehlerquellen für die von anderen Autoren für Meerschweinchenorgane angegebenen Zahlenwerte eine noch größere Rolle spielen — da mit der Feldheimschen Modifikation wenigstens die langsamer reagierenden Störsubstanzen eliminiert werden können — dürfte u. a. eine Erklärungsmöglichkeit für die oft stark abweichenden Befunde im Schrifttum liefern.

Ob diese methodische Fehlerquelle möglicherweise auch in den Rattenorganen eine, wenn auch zweifelsohne geringere Rolle spielt, kann z. Z. nicht mit voller Sicherheit ausgeschlossen werden.

Diskussion

Bisherige Befunde über den Vitamin-C-Gehalt von Tumoren und Tumorträgern

Über den Gehalt von Tumoren an DAS konnten wir im Schrifttum keine Mitteilung finden. Dagegen liegen für AS zahlreiche Befunde vor, die sich allerdings vielfach widersprechen. Erniedrigten Werten in mehr oder weniger deutlicher Ausprägung (47, 161, 164, 178, 146) stehen Angaben gegenüber, die keine deutlichen Unterschiede feststellten (117, 175, 189) oder erhöhte Werte, zumindest den Tumor betreffend, registrierten. Ein Teil der Arbeiten beschränkte sich darauf, nur Tumorgewebe oder nur Organe von Tumorträgern zu untersuchen (29, 131, 177, 193, 194), was naturgemäß die Auswertbarkeit einschränkt, zumal Abweichungen der Untersuchungstechnik und die bereits erwähnten Fehlerquellen der Methodik Vergleiche der Zahlenwerte verschiedener Untersucher nur mit großen Einschränkungen gestatten. Andere Autoren prüften zwar den Vitamin-C-Gehalt von Tumoren im Vergleich zum Normalgewebe des Tumorträgers, ohne indessen auch normale Kontrolltiere zum Vergleich heranzuziehen. Fast allen vorliegenden Veröffentlichungen liegen ferner zu kleine Tierzahlen zugrunde, um eine statistische Auswertung zu ermöglichen. Es würde zuviel Raum beanspruchen, die Vielzahl der vorliegenden, zum großen Teil älteren Publikationen (6, 18, 19, 28, 35, 36, 51, 56, 61, 84, 92, 94, 118, 121, 125, 158, 185, 188, 189, 190, 196) im einzelnen zu diskutieren.

Insgesamt betrachtet, lassen die bisherigen Arbeiten dennoch erkennen, daß die Konzentration an Vitamin C im Tumor absolut und relativ höher ist als der Gehalt an anderen Vitaminen, die meist stark vermindert befunden wurden.

Das Gewicht der Arbeiten, die erhöhte Werte ergaben, dürfte auf Grund des Umfangs des ausgewerteten Materials im allgemeinen höher zu veranschlagen sein als die vorerwähnten Mitteilungen. So betrug in einer Untersuchung von W. V. B. Robertson (147) der Vitamin-C-Gehalt von 22 Tumoren (Maus und Ratte) 15 bis 70 mg pro 100 g Frischgewicht. Im Hepatom 31 der Ratte lag er mit durchschnittlich 32 mg höher als in normaler, regenerierender oder fetaler Leber (Maximum 21 mg/%). Auch in 2 anderen Rattentumoren war mehr Vitamin C enthalten als in Normalorganen (Leber, Milz, Niere), abgesehen von den Nebennieren (189).

Nach M. H. Briggs (31) lag der Vitamin-C-Gehalt in Buttergelbhepatomen bei Bestimmung nach einer anderen Methode mit 25 μg/g ebenfalls höher als in der Normalleber (20 μg/g). Fütterung von Azofarbstoffen und 2-Azetylaminofluoren erhöhte den AS-Gehalt in der Mäuse- und Rattenleber (41), ebenso Injektion kanzerogener Stoffe (48, 94).

Kanzerogene Stoffe scheinen generell die Synthese und Ausscheidung von Vitamin C zu beschleunigen (30, 35, 44, 93, 191).

Über den Vitamin-C-Gehalt menschlicher Tumoren liegen nur relativ wenige Untersuchungen vor. Die meisten Untersucher beschränkten sich auf die Bestimmung des Vitamin-C-Defizits durch AS-Bestimmungen im Urin von Krebskranken nach Zufuhr von Vitamin C.

Die Vitamin-C-Konzentration menschlicher Tumoren zeigt starke Schwankungen, nach einer Untersuchung (70) von Spuren bis zu 4,5 mg%, zum großen Teil in Abhängigkeit von der exogenen Vitamin-C-Zufuhr. Das ist nur etwa ein Zehntel der Werte der Geschwülste Vitamin-C-selbstsynthetisierender Tierarten. In einem Leberkarzinom menschlichen Ursprungs waren nur 0,04 mg/g an Vitamin C enthalten (131).

Über den AS-Gehalt menschlicher Zervikalkarzinome liegt eine neuere japanische Veröffentlichung vor (174). Im allgemeinen ist die Vitamin-C-Menge auch beim Menschen im Tumor höher als im normalen Ausgangsgewebe (198).

Nach einer älteren Untersuchung (1936) an tumortragenden Meerschweinchen enthielt virulentes Sarkomgewebe 0,4 mg/g an AS, nekrotisches Gewebe dagegen fast gar nichts (29).

Physiologische Bedeutung des Vitamin C

Vitamin C fungiert als Wasserstoffüberträger in verschiedenen Reaktionsketten des Stoffwechsels, vor allem beim Abbau von Kohlehydraten, bei der Oxydation von Fettsäuren und im Aminosäurestoffwechsel. Als Oxydationsreduktionssystem ist es dabei an verschiedenen Fermentsystemen beteiligt. In gewissem Sinne stellt es demnach einen Aktivator des Zellstoffwechsels dar.

1. Kohlehydratstoffwechsel

Eine große Zahl von Einzelbeobachtungen liegt hier vor (s. 76, 142), die jedoch noch keinen erschöpfenden Überblick erlauben. Einige davon sind: Beim experimentellen Skorbut ist der Blutzucker erhöht, die Zuckertoleranz, die Leberphosphorylase und der Insulingehalt des Pankreas (β-Zellendegeneration) sind erniedrigt und das Leberglykogen vermindert (13, 15, 130). Bei gleichzeitiger Zufuhr von Glukose und AS ist die Leberglykogenspeicherung größer als bei alleiniger Verabreichung von Glukose (157). Prolongierte Insulinbehandlung führt zur Besserung der Zuckertoleranz und der Glykogensynthese (14). Reichliche Vitaminzufuhr steigert den Effekt der Nebennierenrindenhormone auf Glykogenbildung und -speicherung (11).

2. Aminosäure- und Nukleinsäurestoffwechsel

Der Abbau aromatischer Aminosäuren, insbesondere von Tyrosin und Phenylalanin, in der Leber ist abhängig von der Anwesenheit des Vitamins C (105, 109, 163). Die Einwirkung der AS auf die Tyrosin- und Phenylalaninoxydation scheint Koenzymcharakter zu haben (49, 75, 78, 135). Für die Beteiligung des Vitamins C am Nukleinsäurestoffwechsel spricht ebenfalls eine ganze Reihe von Befunden (62, 64, 152). Der Eiweiß- und RNS-Gehalt der Leber an Skorbut eingegangener Meerschweinchen beträgt weniger als die Hälfte des Normalwertes (57). Das RNS-DNS-Verhältnis von Milz und Nebennieren ist bei Meerschweinchen nach Fütterung einer skorbuterzeugenden Diät ebenfalls verschoben (63).

3. Die Beteiligung der AS an der Biosynthese der Steroidhormone

Vitamin C wird in der höchsten Konzentration von allen Organen in der Nebenniere gefunden. Man nimmt an, daß bei der Oxydation der AS zu Monodehydroaskorbinsäure in der Nebennierenrinde Hydroxylradikale entstehen, die durch Hydroxylasen auf das Steroidmolekül übertragen werden (95, 178). Die Hydroxylierungsfunktion scheint generelle Bedeutung zu haben, z. B. auch bei der Umwandlung von Prolin und Tryptophan in Hydroxyprolin und 5-Hydroxytryptophan unter dem Einfluß von AS (39, 40, 149). Vitamin C ist für bestimmte Oxydationen an Steroidhormonen, z. B. an Desoxykortikosteron, erforderlich (91, 165), ebenso zur Oxydation von Fettsäuren, z. B. von Linolensäure im Gehirn (25, 46). Das letztere scheint allerdings nicht für andere Organe zu gelten. Dem Vitamin C wird ferner eine katalytische Funktion bei der Oxydation von Phospholipoiden in der Rattenleber zugeschrieben (153).

Stresseinwirkungen, die zu einer erhöhten Ausschüttung von Nebennierenhormonen führen, z. B. auch Operationen, Fieber usw., führen zu einem starken Anstieg des Vitamin-C-Verbrauchs. Auch bei Bestrahlungen ist der Bedarf an AS erhöht, wobei offenbleibt, ob das Vitamin C als Reduktionsmittel an der Unschädlichmachung bestimmter toxischer Bestrahlungsprodukte, z. B. von Peroxyden, oder nur indirekt über eine Aktivierung des RES, beteiligt ist. Die Strahlentoleranz ist jedenfalls nach Vitamin-C- (und A-) Gaben gesteigert.

4. Beziehungen zu anderen Vitaminen

AS ist an der Umwandlung von Folsäure zu Folinsäure beteiligt (74, 86, 103, 114, 133). Da Folsäure ebenfalls in den Tyrosinstoffwechsel eingreift, besteht die Möglichkeit, daß die Wirkung auf den Tyrosinstoffwechsel indirekt über die Folsäure erfolgt.

Zwischen Vitamin A und C bestehen gewisse Beziehungen. A-Mangel führt zur Verminderung der AS-Reserven in verschiedenen Organen (89, 97, 116, 176), die sogar bei Tieren, die sonst in der Lage sind, AS selbst zu synthetisieren, solche Ausmaße annehmen kann, daß skorbutähnliche Symptome auftreten (115). Umgekehrt soll bei Mangel an AS die Vitamin-A-Ausscheidung pathologisch erhöht sein, wodurch es sekundär auch zu A-Mangel kommen kann (161). Toxische Dosen an Vitamin A bewirken andererseits einen abnorm niedrigen Vitamin-C-Gehalt in der Nebenniere (37, 151).

Vitamin B_{12} verhindert eine zu rasche Oxydation der AS zu DAS (129).

5. Wirkung auf den Eisenstoffwechsel

Die hypochrome Skorbutanämie wird durch eine Störung des Eisenstoffwechsels bewirkt, die durch AS vermindert werden kann (2, 12, 65, 71, 181).

6. Einfluß auf den Mukopolysaccharidstoffwechsel

Am Stoffwechsel der Grundsubstanz und der kollagenen Fasern ist Vitamin C führend beteiligt (67, 87, 41, 167, 192). Die Kollagenbiosynthese beruht auf der Überführung des Prolins im Präkollagen zu Hydroxyprolin (16, 33, 66, 68, 149, 150). Auch die Synthese der Hyaluronsäure, die zum Aufbau der intrazellulären Kittsubstanz nötig ist, ist Vitamin-C-abhängig. Die bei Skorbut auftretenden petechialen Blutungen hängen damit zusammen.

7. Resorption, Abbau und Ausscheidung

Mechanismus der Wirkung

Die Vitamin-C-Aufnahme erfolgt durch den Dünndarm (104, 137). Im Belastungsversuch am Menschen werden, außer AS, Diketogulonsäure und Oxalsäure als Abbauprodukte ausgeschieden; 5% der applizierten AS (1-C^{14}) lassen sich als respiratorisches CO_2 nachweisen. Beim Meerschweinchen findet man über 60% der zugeführten Dosis im ausgeatmeten CO_2 (32, 81).

Als eigentliche Wirkform der AS wird neuerdings die Monodehydroaskorbinsäure, ein radikalisches Zwischenprodukt bei der Oxydation von AS zu DAS, angenom-

men (101), die durch DPNH · H-Monodehydroaskorbinsäure-Transhydrogenase zu AS zurückgebildet wird. Die Oxydation der AS zu Monodehydroaskorbinsäure wird wahrscheinlich von Zytochrom b_5 katalysiert, wobei noch nicht entschieden ist, ob dieses direkt mit Sauerstoff reagiert (95, 101).

8. Befunde bei Krebs

Vitamin C wird in den wichtigsten endokrinen Drüsen, in jungen, schnell wachsenden Geweben und auch bei der Wundregeneration, in erhöhten Konzentrationen gefunden; zwischen der funktionellen Aktivität des Corpus luteum und dem Vitamin-C-Gehalt besteht ebenfalls ein direkter Zusammenhang. Es wird deshalb vermutet, daß die Bedeutung des Vitamins C beim Krebs in Verbindung mit dem erhöhten Stoffwechsel der Krebszelle zu betrachten sei (124); AS fungiere in der Krebszelle als „Atmungsferment".

Der tägliche Vitamin-C-Verbrauch von Patienten mit vorgeschrittenem Krebs beträgt — nach Absättigung des Defizits — mit 125 mg gegenüber durchschnittlich 67 mg bei den Kontrollfällen etwa das Doppelte (108).

Ein starkes Vitamin-C-Defizit, besonders in späteren Stadien, wurde von zahlreichen Untersuchern festgestellt (3 bis 5, 27, 43, 58, 69, 73, 88, 100, 121, 160 bis 162, 171, 184). G. Gaethgens (58) z. B. verabfolgte bei 20 gynäkologischen Krebsfällen innerhalb von wenigen Tagen bis zu 6000 mg (500 mg täglich), ohne daß der Reduktionswert des Harns anstieg. Bei vielen Krebsfällen konnte das Defizit überhaupt nicht exakt ermittelt werden. W. G. Deucher (43) registrierte Vitamin-C-Defizite bei Krebspatienten zwischen 2500 und 11000 mg (durchschnittlich 4300 mg), E. Schneider (160) im Mittel 4550 mg gegen 1350 mg bei anderen Krankheiten usw. Merkwürdigerweise pflegt man trotz dieses hohen Defizits bei Krebskranken keine skorbutischen Anzeichen zu finden, obwohl die angegebenen Werte das bei Skorbut vorliegende Defizit z. T. weit überschreiten. Gaethgens versucht das Vitamin-C-Defizit durch eine gesteigerte oxydative Zerstörung des Vitamins C im krebskranken Organismus zu erklären.

Der Tumor hat eine höhere Affinität zu Vitamin C als die von uns untersuchten Normalgewebe; das geht daraus hervor, daß die Anreicherung im Tumor wenigstens teilweise auf Kosten der Organe erfolgt (s. Tabelle 1). Die erhöhte Speicherfähigkeit im Tumor dürfte in erster Linie für das Vitamin-C-Defizit verantwortlich sein, d. h., es handelt sich vermutlich zum großen Teil um ein Vitamin-C-Defizit der Geschwulst selbst. Nur so kann das Ausbleiben skorbutischer Erscheinungen angesichts der Höhe der zur Absättigung nötigen AS-Mengen einigermaßen befriedigend gedeutet werden: Die erhöhte Affinität des Tumors zu Vitamin C geht aber offenbar nicht so weit, daß die Vitamin-C-Verschiebungen innerhalb des tumortragenden Organismus zu einer vollständigen Avitaminose der anderen Organe führen. Schneider versucht das Ausbleiben von Skorbutsymptomen damit zu erklären, daß das zugeführte Vitamin C nicht zur direkten Deckung des AS-Defizits verwendet werde, sondern vor allem zur Glykogensynthese.

Ähnlich wie bei Krebspatienten werden auch bei Meerschweinchen die Vitamin-C-Reserven durch einen wachsenden Tumor schnell erschöpft (193).

Es ist nicht anzunehmen, daß der Organismus den Tumor nach Kräften mit Vitamin C versorgt, um dem Tumorwachstum Einhalt zu gebieten, so etwa, wie Antikörper bevorzugt an den Ort des Abwehrkampfes transportiert werden; der schnell wachsende Tumor deckt seinen Vitamin-C-Bedarf vermutlich ähnlich, wie er Muskelgewebe einschmilzt, um seinen Energie- und Baustoffbedarf zu befriedigen. Diese Gegebenheiten muß man sich auch bei therapeutischen Erwägungen mit Vitamin C vor Augen halten. Die Oxydation ist bekanntlich im Tumor durch die meist relativ niedrige Atmung im Vergleich zu der erhöhten Glykolyse (aerobe Glykolyse) unvollständig. Selbst wenn es demnach gelänge, durch Zufuhr von Vitamin C die Atmung zu steigern, würde man wahrscheinlich dem Tumor nur eine zusätzliche Energiequelle erschließen, was kaum einen karzinostatischen Effekt ausüben dürfte. Über Atmungssteigerung in vitro — an Tumorzellen — wird sowohl durch DAS berichtet (80) wie auch über Erhöhung des O_2-Verbrauchs an Leberschnitten normaler und skorbutischer Meerschweinchen durch AS (173). Diese Steigerung war jedoch im Falle der Leber der zugefügten AS-Dosis äquivalent, was darauf schließen läßt, daß sie durch eine Oxydation des zugesetzten Vitamin C selbst verursacht war und nicht durch einen echten Anstieg der Atmung. Mit einiger Reserve muß man vorerst die Herabsetzung der anaeroben Glykolyse von Tumorschnitten in vitro um 34 bis 68 % in Abhängigkeit von der zugesetzten DAS-Menge beurteilen, weil dabei unphysiologisch hohe Dosen verwendet wurden. Außerdem stehen diese Resultate mit den therapeutischen Versuchen der gleichen Autoren an Rattentumoren in gewissem Widerspruch: Hier wurde umgekehrt gerade durch hohe DAS-Dosen kein Effekt erzielt, sondern nur durch vergleichsweise niedrige Mengen.

Die DNS-Synthese in einem virulenten Rattensarkom wird — im Gegensatz zur Rattennormalleber — in vitro durch Zusatz von AS gefördert (62, 63). In Übereinstimmung damit wurde der DNS-Gehalt in einem Meerschweinchensarkom durch skorbutogene Futterzusammensetzung verringert (168).

Eine plausible Deutung eines evtl. Hemmeffekts von Vitamin-C-Zufuhr auf das Tumorwachstum mit unmittelbarem Angriffspunkt an der Krebszelle selbst könnte unter diesen Umständen u. E. bislang nicht gegeben werden. Wo günstige Erfahrungen an Krebspatienten oder Tumortieren gemacht wurden, dürfte es sich um indirekte Wirkungen handeln, um eine Hebung der Abwehrkräfte durch Besserung des Allgemeinzustandes nach Beseitigung des Vitamin-C-Defizits. Ob AS oder DAS dabei verwendet wurde, dürfte nicht entscheidend sein, da in der Leber eine schnelle Regeneration von DAS in AS erfolgt (140). Besonders vor und nach Operationen und bei Röntgenbestrahlung, die schon für sich allein eine Erhöhung des Vitamin-C-Verbrauchs bewirken, dürfte sich eine Beseitigung des Vitamin-C-Defizits empfehlen [s. auch K. H. Bauer (20)]. Andererseits sollte eine Überdosierung an Vitamin A bei Kombination mit der AS-Behandlung im Hinblick auf die erwähnten Befunde vermieden werden. Es läßt sich leider nicht leugnen, daß subjektive Momente bei der Beurteilung klinischer Erfolge in vielen Fällen nicht ohne Einfluß sind. Für das Ausmaß der Entwicklung vieler Krebsformen, speziell in inneren Organen,

fehlen oft klare Kriterien; völlig gleichartig gelagerte menschliche Krebsfälle, die als Kontrolle ohne Zusatzbehandlung dienen könnten, stehen außerdem nur in Ausnahmefällen zur Verfügung. So fehlt es denn auch nicht an unterschiedlichen Auffassungen. Günstige Effekte durch hohe Dosen an Vitamin A und C (2000 mg täglich) mit Hemmung des Krebswachstums und erhöhter Toleranz gegen Röntgenstrahlen sahen E. Schneider (160 bis 162) und v. Wendt (195). Im Hinblick auf die Röntgenbestrahlung wurde andererseits an Lg-Patienten, die während der Bestrahlung einige Wochen bei Vitamin-C-*freier* Diät gehalten wurden, ein besseres Ansprechen als bei den Kontrollfällen beobachtet (123).

Sogar eine Vitamin-C-freie Diät wurde für diesen Zweck angegeben (122).

L. Huber (83) registrierte an 25 schweren gynäkologischen Krebsfällen, die in gleicher Dosis wie durch v. Wendt behandelt worden waren, zwar eine Besserung des Allgemeinzustandes, jedoch keine Beeinflussung des Tumors.

Auffallende Besserung des Allgemeinbefindens und teilweise Rückbildungserscheinungen durch erhöhte Zufuhr an Vitamin A und C an 218 meist inoperablen Krebspatienten stellte ferner H. Reinhold (145) fest. Mehr oder weniger günstige Wirkungen zumindest auf das Allgemeinbefinden durch AS allein oder in Kombination mit A berichten weitere Autoren (7, 8, 22, 23, 98, 126, 128, 143, 156, 179, 187), z. T. auch auf das Blutbild bei Anämie, Leukämie und Bestrahlung (1, 21, 26, 34, 38, 50, 59, 72, 79, 85, 110, 132, 139, 189).

Günstige Beeinflussung des Krankheitsverlaufs bei Krebs wurde auch durch DAS berichtet (9, 24, 111, 112, 155). Bei Experimentaltumoren versuchte man auch Glukoaskorbinsäure (168 bis 170).

Wesentlich größer ist die Zahl der Arbeiten, die eine mehr oder weniger ausgeprägte Stimulierung des Tumorwachstums durch Vitamin C ergab (45, 54, 55, 96, 107, 113, 197).

Leider gestattet auch der größte Teil der bisherigen tierexperimentellen Arbeiten, die einen hemmenden oder beschleunigenden Effekt von Vitamin-C-Verabreichung feststellen, keine statistische Auswertung, zumal der größte Teil dieser Versuche an Tierarten durchgeführt wurde, die selbst Vitamin C synthetisieren können (Ratte und Maus).

Die große Mehrheit der Untersucher konnte keinen deutlichen Einfluß auf die Entwicklung von tierischen Transplantationstumoren konstatieren (10, 17, 42, 82, 99, 106, 120, 127, 134, 136, 138, 144, 172, 198). Schon allein die Tatsache, daß der klinische Verlauf bösartiger Tumoren sich bei Tierarten mit und ohne Vitamin-C-synthetisierender Fähigkeit nicht wesentlich unterscheidet, spricht gegen eine entscheidende Bedeutung sowohl der AS als auch der DAS für das Tumorwachstum.

Bei Skorbutdiät wuchs ein transplantables Fibrosarkom von Meerschweinchen langsamer als bei Normaldiät, wobei der Vitamin-C-Gehalt im Tumor fast auf Null absank (148, 169). In diesem Zusammenhang ist im Auge zu behalten, daß Transplantationstumoren bei Tieren in herabgesetztem Allgemeinzustand ganz allgemein schlechter wachsen.

Eine Reihe weiterer experimenteller Arbeiten seien wenigstens erwähnt (77, 90, 119, 154, 166, 180, 183, 186).

In 5 kleinen Testserien (s. Tabelle 3), in denen Meerschweinchen nach Transplantation des Daels-Sarkoms hohe Vitamin-C-Dosen erhielten, war eine Tendenz zu besserem Tumorwachstum bei den mit AS behandelten Tieren erkennbar.

Tabelle 3 Einfluß von erhöhter Zufuhr an Vitamin C (50 mg täglich) auf die Angangsziffer des Daels-Meerschweinchensarkoms

	Kontrolltiere ohne Zusatzbehandlung mit Vitamin C		mit Vitamin C behandelte Versuchstiere	
	Tierzahl	Angangsziffer	Tierzahl	Angangsziffer
	7	1	4	—
	4	4	4	1
	9	4	10	3
	12	2	6	1
	5	—	4	—
Summe:	37	11 (30%)	28	5 (18%)

Zusammenfassung

In Untersuchungen an 64 Tumor- und Normalratten (5 verschiedene Transplantationstumoren) stellten wir fest:

1. Der Gehalt an Gesamtvitamin C der Tumorgewebe war höher als in den untersuchten parenchymatösen Organen [Leber*, Hirn*, Hoden* und Milz ($p < 5\%$)] der Tumorratten.

2. Die Konzentration an Gesamtvitamin C in Leber*, Milz und Hirn der Tumortiere war niedriger als in den entsprechenden Geweben der Normalratten. Die Anreicherung im Tumor geht demnach mindestens teilweise auf Kosten der Vitaminversorgung der anderen Organe.

3. Die erhaltenen Werte für Gesamtvitamin C in den virulenten Tumoren Yoshida-Sarkom, Walker-Karzinosarkom 256 und Jensen-Sarkom lagen höher als in dem langsam wachsenden Chlorom*.

4. Der Vitamin-C-Gehalt war im ES 4-Sarkom* und Yoshida-Sarkom ($p < 5\%$) mit fortschreitender Tumorentwicklung vermehrt.

5. Bei Meerschweinchen ist die Vitamin-C-Konzentration von den Futterbedingungen abhängig. Reduzierende Störsubstanzen spielen hier bei Verwendung von 2,6-Dichlorphenolindophenol eine größere Rolle als in Rattenorganen. Mit 2,4-Dinitrophenylhydrazin als Indikator weist auch bei Meerschweinchen Tumorgewebe höhere Werte an Gesamtvitamin C auf als Leber ($p < 5\%$), Niere und Hirn; ebenso sind auch die Werte in der Leber der Tumortiere niedriger als in Normalleber ($p < 5\%$).

6. DAS war in allen untersuchten Organgeweben, einschließlich des Tumors, in unterschiedlicher Höhe nachweisbar.

7. Die Grenzen der verwendeten Methodik werden aufgezeigt. Die für DAS gefundenen Werte dürften Minimal-, die für AS festgestellten dagegen Maximalwerte darstellen.

8. Angangsziffer und Wachstum des Daels-Meerschweinchensarkoms wurde durch C-Hypervitaminisierung nicht signifikant beeinflußt; es bestand eher eine Tendenz zur Wachstumsförderung als zur Hemmung des Tumors.

9. Es wird eine Übersicht über die bisherigen Befunde an Mensch und Tier über die Beziehungen zwischen Vitamin C und Tumor gegeben.

Die mit * bezeichneten Schlußfolgerungen sind statistisch gesichert ($p < 1\%$). Für die statistische Auswertung gilt unser Dank Herrn Dipl.-Math. V. Erhardt. Fräulein K. Sachs danken wir für ihre wertvolle technische Mitarbeit.

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Ascorbic Acid, a Coenzyme in Tyrosine Oxidation[1]

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The crystalline antiscorbutic vitamin has been available since its isolation was announced in 1932. However, no adequate explanation of the chemistry of its biological functions has been established. Now at least one of its functions may be understood, for it has been found to be a necessary coenzyme in the metabolic oxidation of the amino acid tyrosine. Following the discovery of its need for complete utilization of tyrosine in the intact animal (*1*), it was shown that liver slices from scorbutic guinea pigs were unable to oxidize tyrosine (as normal slices were) unless the crystalline vitamin were added (*2*). With the *in vitro* addition of crystalline synthetic ascorbic acid, scorbutic liver slices were indistinguishable from normal slices in their ability to oxidize tyrosine as measured by oxygen consumption. Since surviving cells were present, an uncertainty remained as to whether the action of the vitamin was a direct enzymatic participation or some indirect effect.

Studies with cell-free scorbutic liver preparations gave indefinite answers, for with large numbers of animals the average values for tyrosine oxidation by deficient livers were almost equal to the average values obtained with normal livers. In occasional animals, the degree of tyrosine oxidation was increased by the addition of the vitamin. Painter and Zilva (*3*) have observed the increased disappearance of the tyrosine phenolic group with the addition of ascorbic acid to scorbutic liver preparations, which also were capable of extensive tyrosine metabolism.

In contrast, enzyme preparations from even normal animals completely lose the ability to oxidize tyrosine if the preparation is made in such a fashion that the total concentration of both free and bound ascorbic acid is reduced to an insignificant value. This removal of ascorbic acid may be accomplished by thorough dialysis, by certain types of acetone desiccation, by dialysis of other types of acetone powders, and by specific protein fractionations. With preparations of this sort the role of ascorbic acid is readily demonstrated. In order to achieve tyrosine oxidation, it is necessary to add α-ketoglutaric acid as amino group acceptor in the first stage of the reaction. This first stage is probably a nonoxidative transamination, as shown by experiments with tyrosine marked with heavy nitrogen in our own work and by use of ketoglutaric acid (*4*) and other experiments dealing with the transamination enzymes (*5, 6*).

The tyrosine oxidation was carried out by means of the usual manometric procedure, as previously described (*7*). Appropriate solutions of the enzymes combined with the necessary additives were incubated with tyrosine (5 μm), the oxygen consumption observed being corrected by subtraction of suitable controls. With the addition of the ketoglutarate in stoichiometric proportions (5–10 μm) and ascorbic acid in catalytic quantities (150–200 μg), the oxidation of tyrosine occurs with the uptake of 4 atoms of oxygen per mole of tyrosine present. Without the addition of the vitamin the oxidation proves insignificant, with much less than 1 atom of oxygen being consumed. With the total system present in adequate quantity the oxidation proceeds to completion in 1 hr.

From these results it may be argued that ascorbic acid is necessary to the enzyme responsible for the first oxidative step, as may be concluded from the results obtained with liver slices (*2*). In light of this finding it also may be concluded that the vitamin is a coenzyme in this step, a conclusion which is further supported by the finding that the velocity of the oxidation is dependent upon the concentration of ascorbic acid present. In fact, it has been possible to calculate the dissociation constant between the coenzyme and the apoenzyme by the classical procedures of enzyme chemistry (as will be described elsewhere).

Since transamination appears to be the first step in the reaction sequence, it is obvious that *p*-hydroxy-

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phenylpyruvic acid should be regarded as the intermediate involved in the uptake of the first oxygen atom. With this compound substituted for the amino acid and with α-ketoglutaric acid omitted, the same enzyme preparations cause the oxidation of the tyrosine keto acid, provided that sufficient ascorbic acid is added to the system. Without the vitamin insignificant oxidation is obtained. Thus it may be argued that ascorbic acid plays its part in tyrosine metabolism by acting as a coenzyme in the oxidation of the deaminated amino acid.

The exact mechanism of this chemical reaction is unknown, but an attractive working hypothesis may be presented, based upon the reversible enediol oxidation reduction center of the vitamin. The enzyme would be regarded as removing hydrogen from the tyrosine keto acid by means of the dehydroascorbic acid form of the vitamin, with subsequent transfer to oxygen and regeneration of dehydroascorbic acid. Specific experiments testing this hypothesis and more detailed discussion will appear in a subsequent communication.

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7556

Studies in the Stability of Compressed Tablets of Ascorbic Acid

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Ascorbic acid and its salts are known to deteriorate in presence of air. This decomposition is extremely rapid in solution. It is a matter of study whether the compressed tablets of ascorbic acid undergo decomposition in presence of air and whether ascorbic acid tablets require coating in order to preserve the activity of ascorbic acid.

We have studied the decomposition of ascorbic acid tablets in air and our findings are given below.

EXPERIMENTAL

2 grains of compressed tablets containing 50 milligrams of ascorbic acid and lactose (excepient) were prepared with the help of a high compressing rotary tablet machine manufactured by F. J. Stokes. These tablets are kept in a dish and exposed to air. The amount of ascorbic acid was found out by titration with 2.6 dichlorophenol indophenol. The study was continued for six months.

EXPERIMENTAL RESULTS

TABLE I

Amount of ascorbic acid in milligrams

Original	(*a*) 25.5	(*b*) 25.6	(*c*) 25.0
After one month	(*a*) 25.0	(*b*) 25.8	(*c*) 25.4
After two months	(*a*) 25.7	(*b*) 25.5	(*c*) 25.2
After three months	(*a*) 25.8	(*b*) 25.8	(*c*) 25.3
After five months	(*a*) 25.5	(*b*) 25.7	(*c*) 25.0
After six months	(*a*) 25.7	(*b*) 25.4	(*c*) 25.2

CONCLUSION

The compressed tablets of ascorbic acid are stable for fairly long period even in presence of air.

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A Gas Chromatographic Procedure for the Simultaneous Determination of Pyridoxine, Ascorbic Acid, and Nicotinamide in Vitamin Capsules and Tablets

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ALTHOUGH methods have long existed for the determination of pyridoxine, ascorbic acid, and nicotinamide, they are generally rather slow and tedious, and require separate determinations for each compound. The presently accepted United States Pharmacopeia methods for determining pyridoxine, ascorbic acid, and nicotinamide involve coupling with 2,6-dichloroquinonechlorimide, reaction with 2,6-dichlorophenolindolphenol, and reaction with cyanogen bromide, respectively. While accuracies of ±2% and ±3% were reported for pyridoxine (*1*) and ascorbic acid (*2*), results of little better than ±10% can be expected for nicotinamide (*3*). The shortcomings of using a toxic noxious substance such as cyanogen bromide are obvious.

It was desired in this laboratory to devise a method for the rapid simultaneous quantitative determination of pyridoxine, ascorbic acid, and nicotinamide in vitamin capsules and tablets without prior separation of the materials. Gas chromatography seemed the most practical because of its speed and convenience.

While it has been shown in the literature that pyridoxine (*4-6*) and ascorbic acid (*7*) can be readily converted to their TMS (trimethylsilyl) derivatives and subjected to gas chromatography, reported data pertained only to pure samples and application was not made to pharmaceutical preparations.

In the proposed method, BSA [*N,O*-bis(trimethylsilyl)acetamide] is reacted directly with the contents of a multivitamin capsule or tablet and the resulting solution chromatographed, using phenanthrene as internal standard.

EXPERIMENTAL

Chromatographic Equipment and Conditions. A Barber-Colman Model 5340 gas chromatograph equipped with a thermal conductivity detector was used in conjunction with a Hewlett-Packard Model 7127 strip chart recorder. The chart speed of this recorder could be varied from 0.25 to 2.0 inches per minute to facilitate quantitation. The chromatograph was operated isothermally at the following temperatures: injector, 200°; oven, 175°; and detector, 200 °C. The carrier gas was helium flowing at 8 ml per minute. The column was an 8-foot by 1/8-inch o.d. stainless steel coil packed with 15% Dow Corning High Vacuum Silicone Grease on 60–80 mesh Gas Chrom Q.

Sample Preparation. Four methods of sample preparation were found satisfactory:

1. For calibration studies, the desired combination of vitamins were weighed into a volumetric flask.
2. For single tablet assays, it was necessary to finely grind one tablet or the contents of one capsule and transfer the powder into a 5-ml volumetric flask.
3. In the analysis of composites of several tablets or capsules, method 2 can be used on a larger scale—*i.e.*, 10 capsules in 50 ml of BSA.
4. If this were deemed too expensive, it was found equally satisfactory to empty the contents of 8–10 capsules or 8–10 finely ground tablets into a 25-ml volumetric flask. About 20 ml of methanol were added, and the suspension heated about five minutes on a steam bath with swirling to avoid bumping. After coming to room temperature, the final volume was brought to 25 ml. By this time most of the solid material had dissolved, with the remainder not adversely effecting the quantitation. A 3-ml aliquot was taken without attempting to exclude the solid residue. This was transferred to a 5-ml volumetric flask, and the methanol completely evaporated under a stream of nitrogen.

About 20 mg of accurately weighed phenanthrene were added to the solid material prepared by any of the above methods. Three milliliters of BSA were also added, the flask stoppered, and allowed to stand at room temperature. The initial reactions taking place were exothermic, causing the temperature to rise somewhat. After returning to room temperature (about 10 minutes), the flask and its contents were heated for approximately five minutes at 85 °C on a sand bath to complete reaction of ascorbic acid with BSA. After again returning to room temperature, the contents were diluted to volume with acetonitrile or BSA, shaken, and a 5-μl aliquot taken for injection into the chromatograph. This sample size was chosen to give a pyridoxine peak of sufficient size for accurate quantitation; it would not be necessary with a more sensitive detector.

Quantitation. Two methods were tested for measuring peak areas: cut-and-weigh and area measurement with a planimeter. The latter method gave the most consistent and reliable results. Each sample was injected thrice and results were determined by averaging the figures thus obtained.

To determine the weight sensitivity constants (K) of the thermal conductivity detector to each of the vitamins of interest (relative to phenanthrene), ten samples were weighed with the following compositions: 50 mg nicotinamide, 300 mg ascorbic acid, 2 and 10 mg pyridoxine; 300 mg ascorbic acid, 5 mg pyridoxine, 20, 80, 100, and 150 mg nicotinamide; 50 mg nicotinamide, 5 mg pyridoxine, 50, 100, 200, and 400 mg ascorbic acid. Approximately 20 mg of phenanthrene were accurately weighed into each sample. These were silanated, injected in triplicate, and K determined according to the following formula:

$$K = \frac{\text{(mg of phenanthrene in sample) (area of vitamin peak)}}{\text{(area of phenanthrene peak) (mg of vitamin in sample)}}$$

Although the vitamins were analyzed as their trimethylsilyl derivatives, all calculations were made as though the parent compounds were being chromatographed.

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After K had been determined, a simple rearrangement of the formula was used to determine the amounts of each of the vitamins of interest in the flask from which the injection had been taken. When the methanol extraction method was used for sample preparation, the answer obtained was multiplied by a factor of:

$$\frac{[\text{size of volumetric flask used for extraction (in ml)}]}{[\text{number of tablets or capsules originally used}] \times [\text{size of aliquot (in ml)}]}$$

Because of the great differences in the amounts of the components of the tablets and capsules—*i.e.*, small amount of pyridoxine and great amount of ascorbic acid—it was necessary to vary the attenuation of the chromatograph for each peak (see Figure 1).

RESULTS AND DISCUSSION

Using the proposed method, the following relative weight sensitivity factors were obtained: $K_{\text{nicotinamide}} = 1.63 \pm 0.06$, $K_{\text{pyridoxine}} = 1.45 \pm 0.05$, and $K_{\text{ascorbic acid}} = 1.91 \pm 0.04$. The fact that the values of K were consistent for all the samples used indicated that the response of the detector was linear throughout the ranges 20–150 μg nicotinamide, 2–10 μg pyridoxine, and 50–400 μg ascorbic acid. It also indicated that varying concentrations of vitamins did not affect the results.

Although the column was overloaded with ascorbic acid–TMS throughout the 50–400-μg range (as indicated by the leading of its peak), the quantitation was consistent and the ascorbic acid–TMS was completely separated from pyridoxine–TMS on the chromatogram (see Figure 1).

Two brands of vitamin capsules were purchased and their nicotinamide, pyridoxine, and ascorbic acid contents determined using methods of the current United States Pharmacopeia. These results were compared with those obtained using the proposed gas chromatographic method.

A commercial preparation was obtained which contained all the ingredients of a commercial vitamin tablet except pyridoxine, ascorbic acid, and nicotinamide. To this placebo were added accurately weighed amounts of the vitamins of interest. This simulated preparation was then gas chromatographically analyzed in triplicate, and the results obtained were compared with actual values. Results of this and the above tests are summarized in Table I.

For the proposed procedure the use of hexamethyldisilazane was found to be unsatisfactory for silanation. It has been reported in the literature that this reagent tends to incompletely silanate ascorbic acid (*7*). It was found in this laboratory that hexamethyldisilazane also incompletely silanated nicotinamide. When it was used, the nicotinamide peak in the chromatograms appeared as a doublet, with the two component peaks of varying relative size. It was assumed that the earlier peak represented the monosilanated *N*-trimethylsilylnicotinamide, and the latter one *N,O*-bis(trimethylsilyl)nicotinamide, for the second one increased as the first one decreased in intensity with time. Hexamethyldisilazane also tended to produce a precipitate of ammonium chloride upon

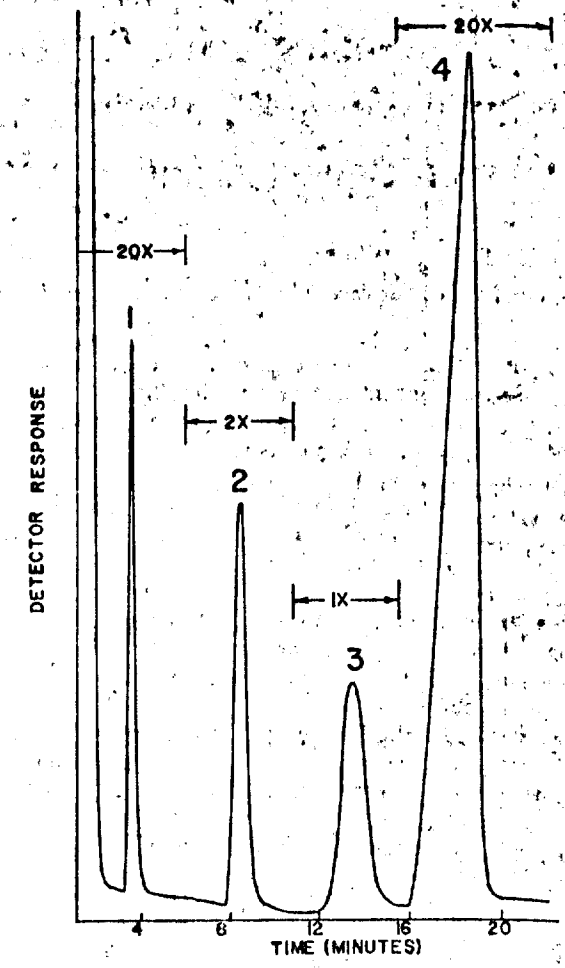

Figure 1. Chromatogram of a vitamin preparation

1, Nicotinamide–TMS, *2* Phenanthrene, *3*, Pyridoxine–TMS, *4*, Ascorbic acid–TMS. Chart speed = 0.25 inches per minute

Table I. Analysis of Commercial and Simulated Preparations

	Component	Amount declared or actual (mg/cap)	% Found of declared or actual	
			GC method	USP method
Brand A	Nicotinamide	50	98	106
	Pyridoxine	5	106	108
	Ascorbic acid	300	107	109
Brand B	Nicotinamide	50	102	112
	Pyridoxine	5	105	106
	Ascorbic acid	300	110	108
Commercial placebo	Nicotinamide	100	97	
	Pyridoxine	5	99	
	Ascorbic acid	200	98	

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Effect of Acetylsalicylic Acid and Ascorbic Acid on Oxygen Toxicity

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SERRILL, S., D. JEFFERSON, J. QUICK and C. E. MENGEL. *Effect of acetylsalicylic acid and ascorbic acid on oxygen toxicity.* Aerospace Med. 42(4):436-438. 1971.

With the use of hyperoxic environments in space capsules and for medical purposes, this study explored the effects of some common drugs with redox potential on oxygen toxicity. Acetylsalicylic acid and ascorbic acid were injected into chow-fed and tocopherol-deficient mice. In vitro lysis of RBCs by H_2O_2 increased from 40 ± 2% to 64 ± 7% in chow-fed and from 60 ± 3% to 90 ± 2% in tocopherol-deficient mice, 60 minutes following injection. A linear response between drug dose and H_2O_2 lysis existed. During exposure to 100% O_2 at 45 psia, drug-treated and tocopherol-deficient mice began seizures and died much sooner than did controls. These data (1) show increased RBC lytic sensitivity to H_2O_2 from mice treated with ASA and ascorbic acid, (2) demonstrated untoward effect of these drugs on in vivo hyperoxic exposure, (3) illustrate predictive use of in vitro H_2O_2 lysis for in vivo toxicity and (4) suggest some commonly ingested drugs could be detrimental to humans exposed to significant hyperoxia.

THE USE OF HYPEROXIC environments for space travel[5] and medical purposes[1] intensified the need to identify factors which might influence susceptibility to oxygen toxicity. Our previous studies have shown that exposure of susceptible humans[6] and tocopherol-deficient mice[2] to 100% oxygen under high pressure (OHP), caused peroxidation of red blood cell lipid resulting in hemolysis. Supplementation of mice with α-tocopherol, an inhibitor of lipid peroxidation, prior to OHP exposure, prevented hemolysis. Tocopherol status of these animals also influenced the occurrence of seizures and death in mice exposed to OHP. It was also proved in mice[2] and implied in man[6] that RBC lytic sensitivity to hydrogen peroxide (H_2O_2) *in vitro*, predicted *in vivo* susceptibility to oxygen toxicity. Some preliminary data from our laboratory indicated that red cells from humans showed increased lytic sensitivity to H_2O_2 *in vitro*, after ingestion of the commonly used drugs acetylsalicylic acid (ASA) and ascorbic acid (vitamin C). The present studies were undertaken to examine the effects of these drugs on susceptibility to oxygen toxicity in mice.

MATERIALS AND METHODS

Animals—Male and female mice, strain DBA/2,* four to six months old and of comparable size (average weight 22 gms) were used. Study groups included mice fed either a tocopherol-deficient test diet** for a minimum of six weeks, or a standard chow diet. Tocopherol-deficient and chow-fed animals were also studied after tocopherol supplementation (1 mgm vitamin E intraperitoneally 12 hours and 1 hour prior to study).

Study of Red Blood Cells—Ether-anesthetized mice were exsanguinated by severing axillary blood vessels. Blood was collected from each animal with heparinized pipettes and placed in separate test tubes maintained at 4°C. *In vitro* lytic sensitivity of erythrocytes to hydrogen peroxide (H_2O_2) was determined on each individual sample, by methods previously described.[4] A concentration of .075% H_2O_2 was used for all determinations.

Exposure to Hyperbaric Oxygen—Groups of four or five mice were exposed to 100% oxygen in a cylindrical hyperbaric chamber (16 inches long, 6 inches internal diameter, total volume 450 inches) equipped with a 6-inch diameter viewing porthole.† The floor of the chamber was covered with a one-inch layer of soda lime USP, to absorb carbon dioxide. Each exposure included: 5 minutes of gas flush with 100% oxygen at 15 psia (pounds per square inch absolute); a 15-minute stepwise compression period to final pressure of 45 psia, maintained with a continuous gas flsh of 100% oxygen at a rate of 15 L/min. Animals were observed through-

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*DBA/2 mice were supplied by the Jackson Laboratory, Bar Harbor, Maine.

**Tocopherol deficient test diet, General Biochemicals, Chagrin Falls, Ohio.

†Table Top Model No. 614, The Bethlehem Corp., Bethlehem, Pennsylvania.

out the entire exposure until death occurred. The times of initial convulsion (defined as generalized tonic, clonic seizures) and death for each animal were recorded.

Drugs—Mice were injected intraperitoneally with either ASA, vitamin C or an equal mixture of both drugs. Prior to each study, freshly prepared drug solutions were made as follows: Powdered ASA or ascorbic acid was mixed with deionized distilled water to a final concentration of 100 mgm/ml. The combined drug mixture was prepared by mixing equal amounts of each drug together with deionized, distilled water to a final concentration of 100 mgm/ml for each drug.

Format of Studies—In initial dose response studies chow-fed mice were given from 1 mgm to 40 mgm of each drug in combination and H_2O_2 lysis of red cells was determined 20 minutes following drug injection. Red cell H_2O_2 lysis was then determined for chow-fed, tocopherol-deficient and tocopherol-supplemented mice at 10, 20, 60 minutes and 24 hours, following injection of 10 mgm of each drug either singly or in combination. Animals given both drugs in combination were exposed to OHP and seizures and death were recorded. Appropriate control animals at room air were used for all studies.

RESULTS

The effect of varying doses of the combined drugs on RBC, H_2O_2 lysis in chow-fed mice is shown in Figure 1. There is an increase of RBC lysis by H_2O_2 above control values as the drug dose is increased. Levels of the drug beyond 40 mgm were consistently lethal. The dose of 10 mgm for each drug was then selected for all subsequent studies.

The effect of combined drugs on RBC H_2O_2 lysis in chow-fed mice with time is shown in Figure 2. Within 10 minutes after drug injection there was a significant and consistent increase in the lytic sensitivity of erythrocytes which lasted for up to 60 minutes. Levels of red cell lysis returned to normal by 24 hours.

The effect of combined drugs on H_2O_2 lysis of red cells in tocopherol-deficient mice is seen in Figure 3. In contrast, to simultaneous tocopherol-deficient control animals whose values remained constant throughout the study period, there was an abrupt increase in lytic sensitivity 10 minutes after drug injection from 60% lysis to 80% RBC lysis. This increased even more at 20 minutes to 93%. After 24 hours the percent H_2O_2 lysis in tocopherol-deficient mice was still elevated significantly above control values. This is in contrast to results obtained in chow-fed animals (Figure 2).

Lysis of red cells by H_2O_2 in chow-fed and tocopherol-deficient mice was decreased to 0% by prior supplementation with tocopherol.

Administration of single drugs at the 10 mg and 20 mg level did not result in the increased lysis that occurred with both drugs in combination.

The effect of hyperoxia and the drug combination on seizures in mice is shown in Table I. In contrast to chow-fed mice, chow-fed animals given drug had a sig-

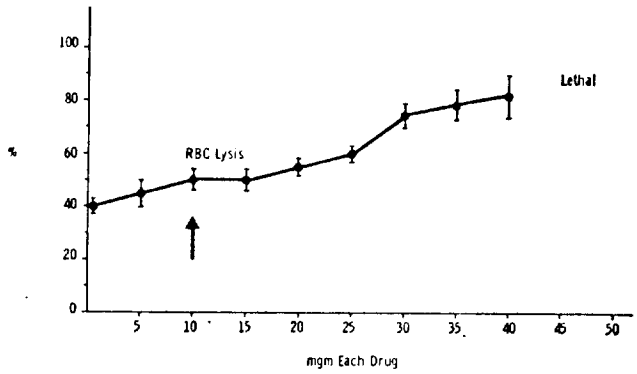

Fig. 1. The effect of ASA (acetylsalicylic acid) and ascorbic acid on red cell lysis by H_2O_2 in chow-fed mice. The points represent means and bars represent 1 S.D. of 5 animals at each drug dose level. The arrow indicates the drug dose selected for all subsequent studies.

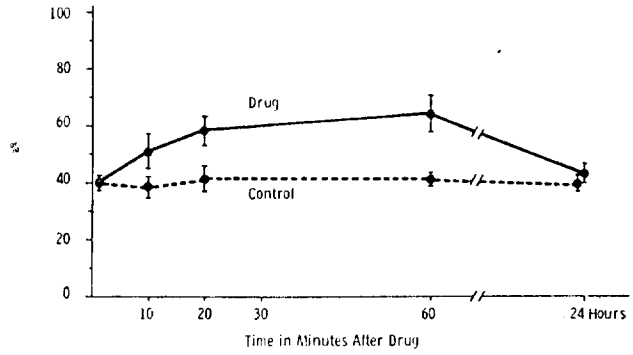

Fig. 2. The effect of combined acetylsalicylic acid and ascorbate on H_2O_2 lysis of cells in chow-fed mice. Points indicate means and bars 1 S.D. of 10 to 25 mice at each point.

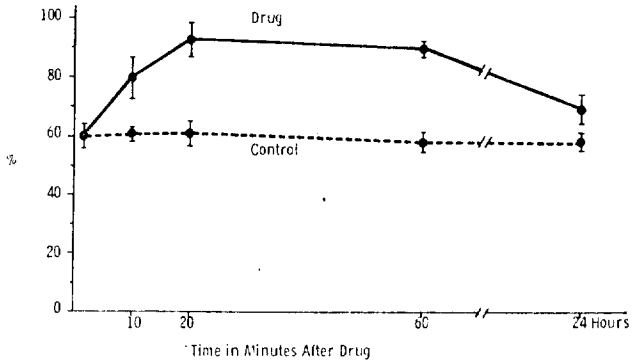

Fig. 3. The effect of combined acetylsalicylic acid and ascorbate on H_2O_2 lysis of red cells in tocopherol-deficient mice. Points indicate means and bars 1 S.D. of 10 to 25 mice at each point.

TABLE I. THE EFFECT OF COMBINED DRUG ADMINISTRATION ON SEIZURES OCCURRING DURING EXPOSURE TO OHP

Study Group of Mice (30 each)	Time to Onset of Seizures During OHP in Minutes (Mean ± 1 S.D.)
Chow Fed	90 ± 10
Chow Fed and Drugs	75 ± 7
Tocopherol Deficient	70 ± 10
Tocopherol Deficient and Drugs	57 ± 6
Tocopherol Supplemented	180 ± 20
Tocopherol Supplemented and Drugs	174 ± 22

TABLE II. THE EFFECT OF COMBINED DRUG ADMINISTRATION ON MORTALITY OF MICE EXPOSED TO OHP

Study Group of Mice (25 each)	Time to Death During OHP in Minutes (Mean ± 1 S.D.)
Chow Fed	270 ± 49
Chow Fed and Drugs	190 ± 21
Tocopherol Deficient	216 ± 20
Tocopherol Deficient and Drugs	155 ± 17
Tocopherol Supplemented	480 ± 60
Tocopherol Supplemented and Drugs	506 ± 75

nificant decrease in time to onset of seizures. As noted they behaved more like tocopherol-deficient mice. When tocopherol-deficient mice were given drug, the time to onset of seizures was even more markedly shortened. Tocopherol-supplemented mice were relatively resistant to seizures. Drug administration to tocopherol-supplemented mice had no untoward effect.

The effect of hyperoxia and drug administration on mortality is shown in Table II. In contrast to chow-fed mice, death occurred more quickly in chow-fed mice given drugs. These values are in a range similar to those of tocopherol-deficient mice. Drug administration to tocopherol-deficient mice resulted in an even greater shortening of survival. Tocopherol supplementation protected mice for extensive periods and completely protected against any drug effects.

DISCUSSION

The use of slightly hyperoxic environments for space travel and the use of oxygen under high pressure for medical purposes has intensified, in recent years, the need to identify factors which might influence susceptibility to oxygen toxicity.

In previous studies from our laboratory, we had demonstrated tocopherol deficiency as one factor that would increase susceptibility of humans and animals to 100% oxygen under high pressure. Under such circumstances, peroxidation of red cell lipid resulted in hemolysis. It was also shown in mice (and implied in man) that red cell lytic sensitivity to hydrogen peroxide *in vitro* predicted the *in vivo* susceptibility to oxygen toxicity.

Since some preliminary data from our laboratory indicated that red cells from humans showed increased lytic sensitivity to hydrogen peroxide *in vitro* after the ingestion of some commonly used drugs (acetylsalicylic acid and ascorbic acid) the present studies were initiated to examine the effect of these drugs on susceptibility to oxygen toxicity in mice.

The studies reported herein clearly demonstrate a dose relationship between the amount of drug given and *in vitro* sensitivity of red cells to hydrogen peroxide. In addition, it was demonstrated that administration of such drugs increased sensitivity of animals to oxygen toxicity as reflected by onset of seizures and mortality. The concomitant presence of tocopherol deficiency accentuated the sensitivity and such sensitivity was reduced by the administration of tocopherol. The mechanism of this drug effect remains obscure. However, it is well known that these agents may produce hemolytic anemia in individuals deficient in the red cell enzyme glucose-6-PO_4-dehydrogenase.[6] This presumably related in some way to the defective antioxidant capabilities of these cells with inadequate mechanisms involved in the hexose-monophosphate shunt. Presumably, this is ultimately related to the failure to produce adequate quantities of reduced glutathione.

Small quantities of endogenously produced hydrogen peroxide in red cells are presumably detoxified by the enzyme glutathione peroxidase which requires adequate quantities of reduced glutathione. Any defect in the hexose-monophosphate shunt results in enhanced susceptibility to agents which produce hydrogen peroxide. Similarly, agents which produce excessive hydrogen peroxide might be expected to increase the stress on this shunt. If tocopherol, indeed, has a role in hydrogen peroxide detoxification (as has been previously suggested by other studies in this laboratory) then such animals might be expected to be more susceptible to the untoward effects of these drugs.

It is possible, therefore, that ascorbic acid and acetylsalicylic acid increase the endogenous formation of hydrogen peroxide which results in an unusual oxidant stress to these cells. Lacking total antioxidant capability (in this case reflected by tocopherol deficiency), changes might then occur not only in red cells, but also in other tissues including brain.

Although the exact mechanisms of these effects remain somewhat obscure, these studies do establish the fact that some commonly ingested drugs such as acetylsalicylic acid and ascorbic acid may influence clinical susceptibility to the untoward effects of oxygen. They also illustrate the predictive nature of the *in vitro* H_2O_2 red cell lysis test.

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ANAPHYLACTIC SHOCK DUE TO SENSITIZATION TO ASCORBIC ACID

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Ascorbic acid (vitamin C) is known in medicine as an agent that takes part in regulation of the oxidation-reduction processes, carbohydrate metabolism, capillary permeability, blood coagulation, tissue regeneration, and formation of steroid hormones. It is not synthesized in the body and is therefore widely used whenever intensified utilization is proposed, for example, in marked physical exertion, mental stress, pregnancy, infections and poisonings, healing of wounds and fractures, etc. Ascorbic acid is effective in the treatment of scurvy.

Ascorbic acid is quickly absorbed through the gastrointestinal tract and well distributed in the tissues. It is quickly excreted by the kidneys. Ascorbic acid has an acid taste and in large doses irritates the oral mucosa. It also depresses the functioning of the islands of Langerhans, injures the renal glomeruli, inducing a toxic hypertensive nephropathy, and causes insomnia (M. D. Mashkovskiy; Schettler).

Ascorbic acid is little known as a factor that causes sensitization, [redacted]. Salén observed extensive rashes and positive skin and intradermal tests in a bronchial asthma patient who came in contact with ascorbic acid. Panzani noted in a bronchial asthma patient an immediate reaction and positive transport of antigens by the method of Prausnitz and Küster. The follow is the case history of a woman who died of anaphylactic shock after receiving an intramuscular injection of ascorbic acid.

Patient K., born in 1929, suffered a long time from periodontosis for which she was frequently treated BEGINNING IN December 1964 with topical agents (oxygen, aloe, maraslavin) and general analeptics (vitamins and aloe parenterally) with a transient effect.

Moreover, she had been repeatedly treated since 1962 for a variety of diseases - follicular tonsillitis (twice), inflammation of the upper respiratory tract, exacerbations of chronic cholecystitis and normacid gastritis, sacrolumbar radiculitis, cardiac neurosis, and misdiagnosed rheumatism. Thus, she had received different drugs, including bicillin (twice), chloramphenicol, sulfanilamides, aspirin, dipyrone, etc.

The patient first showed signs of increased sensitivity to drugs in November 1968 during the treatment of periodontosis by parenteral injection of aloe: deterioration in the sense of well-being, abdominal crises, and hives almost after every injection. These phenomena were regarded as a manifestation of drug sickness and AS A RESULT aloe treatment was suspended and no longer used. All the allergic symptoms disappeared without further treatment. However, similar phenomena began to appear after she DYED her hair or used detergents. She therefore stopped using them.

A course of treatment of periodontosis was started in June 1971 with the application of maraslavin to the gums, ingestion of calcium chloride, and parenteral injection of vitamin C (1 ml of a 5% solution). The injections were given daily except Sundays and some Saturdays; the technique was correct and there were no complications. The first 3 or 4 injections were well tolerated, but the next 2 or 3 resulted in brief abdominal crises that passed in 2 to 3 hours without additional medication. The patient did not bring these crises to the attention of the physician or other medical personnel. Some 5 to 10 minutes after the 7th injection, which was given on the 17th day of treatment, she suddenly felt ill. Then followed suffocation, sharp pain in the epigastric

region, some vomiting, loss of consciousness, involuntary urination and defecation. Resuscitation measures were unsuccessful and she died 15 minutes later without regaining consciousness.

Forensic medical examination revealed signs of acute emphysema and pulmonary edema, bronchorrhea, swelling and edema of the brain with strangulation, acute degeneration of the heart and lungs, venous hyperemia of the viscera, and liquid blood in the large vessels.

Microscopic examination of the organs revealed signs of impaired peripheral blood circulation, especially in the brain, lungs, liver, and spleen, acute degenerative changes in the nerve cells and edema of the interstitial substance of the brain, and acute interstitial edema and foci of acute emphysema in luhg tissue.

Forensic chemical examination of the blood and viscera failed to turn up any toxic substances. The erroneous injection of other substances and air embolism were ruled out.

The foregoing data suggested that the 42-year-old women sensitized to a number of chemical substances died of anaphylactic shock THAT developed acutely after an intramuscular injection of ascorbic acid to which she had apparently become highly sensitized in the course of treatment. This conclusion was confirmed by the allergic anamnesis of the patient, time she went into shock, characteristic clinical picture with abdominal crises, sudden suffocation, and death within a few minutes after the preparation was injected. The resuscitation measures failed because of the severity of the shock and presence of a drug (antigen) in muscular tissue, which precluded the possibility of blocking its effect on the body.

It is evident from an analysis of all the circumstances of this case that there is a lack of strict indications for the use of ascorbic

acid by intramuscular injection in persons exhibiting distinct signs of altered sensitivity to drugs and other chemical agents. We therefore consider it necessary once again to caution physicians against the routine prescription of drugs, especially for parenteral administration. The case described shows that the haphazard use of highly antigenic preparations helped to sensitize the patient not only to these particular drugs but also to a simplar chemical substance, ascorbic acid.

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ЗАМЕТКИ И НАБЛЮДЕНИЯ ИЗ ПРАКТИКИ

УДК 616-001.36-056.3-02:615.356:577.164.2

АНАФИЛАКТИЧЕСКИЙ ШОК ВСЛЕДСТВИЕ СЕНСИБИЛИЗАЦИИ К АСКОРБИНОВОЙ КИСЛОТЕ

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Аскорбиновая кислота (витамин С) известна в медицине как средство, принимающее участие в регуляции окислительно-восстановительных процессов, углеводного обмена, проницаемости капилляров и свертываемости крови, в регенерации тканей и образовании стероидных гормонов. Аскорбиновая кислота не синтезируется в организме, поэтому ее широко применяют во всех случаях, когда предполагается ее усиленное использование, например при значительной физической нагрузке, умственном напряжении, беременности, при инфекциях и интоксикациях, при заживлении ран и переломов и т. д. Как эффективное лечебное средство аскорбиновая кислота применяется при цинге.

Аскорбиновая кислота быстро всасывается через желудочно-кишечный тракт, хорошо распределяется в тканях и быстро выводится почками.

В больших дозах аскорбиновая кислота, имея кислый вкус, раздражает слизистые оболочки ротовой полости, оказывает угнетающее действие на функции островков Лангерганса поджелудочной железы, повреждает почечные гломерулы с развитием токсической гипертонической нефропатии и вызывает нарушение сна (М. Д. Машковский; Schettler).

Как фактор, вызывающий сенсибилизацию организма, аскорбиновая кислота известна мало. Salén наблюдал распространенную сыпь и положительные кожные и внутрикожные пробы у больной бронхиальной астмой при контакте с аскорбиновой кислотой. Panzani у больного бронхиальной астмой отметил реакцию немедленного типа и положительный перенос антител по методу Праусница — Кюнстера. Приводим наблюдение, касающееся женщины, умершей от анафилактического шока после внутримышечной инъекции аскорбиновой кислоты.

Больная К., 1929 г. рождения, длительно страдала пародонтозом, по поводу которого, начиная с декабря 1964 г., ей неоднократно проводилось лечение местнодействующими (кислород, алоэ, мараславин) и общеукрепляющими (витамины и алоэ парентерально) средствами с временным эффектом.

Помимо того, с 1962 г. она неоднократно лечилась по поводу различных заболеваний — фолликулярной ангины дважды, катара верхних дыхательных путей, обострения хронического холецистита и нормацидного гастрита, пояснично-крестцового радикулита, невроза сердца, а также ошибочно предполагавшегося ревматизма. В связи с этим больной назначали различные препараты, в том числе неоднократно бициллин, однократно левомицетин, сульфаниламиды, аспирин, анальгин и др.

В ноябре 1968 г. во время лечения пародонтоза парентеральным введением алоэ у больной впервые появились признаки повышенной чувствительности к лекарству: плохое самочувствие, абдоминальные кризы и крапивница почти после каждой инъекции. Эти состояния были расценены как проявление лекарственной болезни, в связи с чем лечение алоэ было прекращено и в последующем не применялось. Все явления аллергии прошли без дополнительного лечения. Однако в дальнейшем аналогичные состояния стали возникать после окраски волос и применения стиральных порошков, в связи с чем больная перестала пользоваться этими средствами.

В июне 1971 г. был начат очередной курс лечения пародонтоза смазыванием десен мараславином, приемом хлористого кальция внутрь и парентеральным введением витамина С (по 1 мл 5% раствора). Инъекции производили ежедневно, исключая воскресные и отдельные субботние дни, технически правильно и без осложнений. Первые 3—4 введения больная перенесла хорошо. Следующие 2—3 введения сопровождались краткопременными абдоминальными кризами, которые проходили часа через 2—3 без дополнительного введения лекарств. О появлении этих кризов больная не поставила в известность врача и медицинский

персонал. Через 5—10 мин. после очередной, 7-й, инъекции, произведенной на 17-й день лечения, у нее внезапно резко ухудшилось самочувствие, появились удушье, сильные боли в эпигастральной области, необильная рвота, потеря сознания, непроизвольные мочеиспускание и дефекация. Меры реанимации оказались неэффективными. Через 15 мин., не приходя в сознание, больная умерла.

При судебно-медицинской экспертизе выявлены признаки острой эмфиземы и отека легких, бронхорея, набухание и отек головного мозга с нижним ущемлением, острая дистрофия сердца и печени, венозное полнокровие внутренних органов, жидкая кровь в крупных сосудах.

При микроскопическом исследовании органов обнаружены признаки расстройства периферического кровообращения, наиболее выраженные в мозге, легких, печени и селезенке, острые дистрофические изменения в нервных клетках и отек межуточного вещества мозга, острый интерстициальный отек и очаги острой эмфиземы в ткани легких.

При судебно-химическом исследовании крови и внутренних органов умершей токсически действующих веществ не обнаружено. Ошибочное введение других веществ и воздушная эмболия исключены.

Приведенные данные позволили сделать заключение о том, что женщина 42 лет, сенсибилизированная к ряду химических веществ, умерла от анафилактического шока, остро развившегося после внутримышечного введения аскорбиновой кислоты, к которой, по-видимому, в процессе лечения у больной развилась повышенная чувствительность. Подтверждают это предположение аллергический анамнез больной, сроки развития шока, характерная клиническая картина с абдоминальным кризом, приступом внезапного удушья и скоропостижной смертью в течение нескольких минут после инъекции препарата. Неэффективность реанимационных мер обусловлена, возможно, не только тяжестью развившегося шока, но и наличием лекарства — антигена — в мышечной ткани, что исключало возможность устранения его действия на организм.

При анализе всех обстоятельств развития данного заболевания обращает на себя внимание отсутствие достаточно строгих показаний к применению аскорбиновой кислоты путем внутримышечных инъекций у лица, имеющего отчетливые проявления измененной чувствительности организма на лекарства и некоторые другие химические вещества. Поэтому мы считаем нужным еще раз обратить внимание врачей на необходимость избегать шаблонных назначений медикаментов, особенно парентерально. Как видно из приведенного примера, беспорядочное применение высокоантигенных препаратов способствовало развитию сенсибилизации организма не только к этим лекарствам, но и к более простым химическим веществам, каким является аскорбиновая кислота.

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Studies in Experimental Tuberculosis: Effect of Reduced Food Consumption in Infected Guinea-pigs on Carbohydrate Metabolism

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Ascorbic acid levels in tissues of tuberculous guinea-pigs and the effect of reduced food consumption on glycogen and protein contents, and the activities of glycogen phosphorylase, glucose-6-phosphatase and fructose-1-6-diphosphatase have been studied. A significant decrease in ascorbic acid content of liver, spleen, kidney and heart was observed. It is suggested that there is an enhanced utilization of ascorbic acid in experimental tuberculosis. Reduced food consumption by the tuberculous guinea-pigs contributed towards reduced glycogen content, reduced phosphorylase activity and increased fructose-1-6-diphosphatase activity of liver, whereas the protein content was unaffected. Glucose-6-phosphatase activity increased in the liver of pair-fed animals.

DISTURBANCES in carbohydrate metabolism have been observed in experimental tuberculosis in guinea-pigs. Singh *et al.*[1] and Bhargava *et al.*[2] observed decreased glycogen content in the liver of tuberculous guinea-pigs as well as enhanced activities of glucose-6-phosphatase (per 100 g. body weight) and fructose-1-6-diphosphatase. Starvation has also been known to produce similar effects in the animal. In our earlier experiments with tuberculosis, it was observed that the food consumption of the infected animals was very much reduced as compared to the controls. Four weeks after infection and at the time of sacrifice, the intake of food by the tuberculous animals was about half that of the food taken by the *ad libitum* controls. It was felt that this drastic reduction in food consumption by the infected animals might also affect the carbohydrate metabolism to some extent, especially glycogen content, glucose-6-phosphatase and fructose-1-6-diphosphatase activities. The present investigation was undertaken to study the extent to which the reduced food consumption in the infected animals was responsible for the changes in carbohydrate metabolism.

In recent years, the levels of various dietary constituents in blood are taken as an index of nutrition in tuberculosis. Lowered values were conspicuous for ascorbic acid in blood in tuberculosis according to some workers[3]. Severity of tuberculosis was found to parallel reduced ascorbic acid content in blood. However, there are very few reports regarding quantitative measurement of ascorbic acid in tissues in experimental tuberculosis. Since ascorbic acid, in general, is known to play a role in resistance to infection, it was interesting to investigate the tissue levels of this vitamin in tuberculous guinea-pigs. A preliminary report on levels of ascorbic acid in tuberculous guinea-pigs has already been published[4].

In the present study, the effect of pair feeding on glycogen and protein contents, and the activities in liver of phosphorylase, glucose-6-phosphatase and fructose-1-6-diphosphatase were studied. Ascorbic acid contents of liver, spleen, kidney and heart were estimated in tuberculous and pair-fed control guinea-pigs.

Materials and Methods

Male guinea-pigs weighing 300-500 g. were divided into three groups on the basis of age and weight. Male guinea-pigs were selected in preference to female ones to avoid disturbances in metabolism due to hormonal activity. One group was injected intraperitoneally with 0·1 mg. dry weight of tubercle bacilli $H_{37}Rv$ obtained from the National Collection of Type Cultures, London. Of the remaining two groups, one group was fed *ad libitum* and the other was pair-fed. The pair-fed controls were given the same amount of food which the tuberculous animals consumed. The diet consisted of germinated gram (25 per cent), barley (25 per cent) and wheat bran (45 per cent); the rest 5 per cent was made up of common salt, calcium carbonate and shark liver oil. All the animals were sacrificed at the end of 4 weeks, and the tissues were processed for chemical and enzymatic assays.

Estimation of ascorbic acid — This was done according to the method of Maickel[5]. Liver, spleen, kidney and heart tissues were homogenized in ice-cold 4 per cent trichloracetic acid and the homogenates were centrifuged in the cold to remove the proteins. To 1 ml. of the supernatant were added 5·0 ml. of 0·5 per cent aqueous α-α′-dipyridyl and 1 ml. of 1 per cent aqueous ferric chloride. Optical density was measured in the tubes after 10 min. at 525 mμ. With each experiment a freshly prepared standard containing 40 μg. ascorbic acid in 4 per cent trichloracetic acid was taken. The results are expressed in terms of mg. ascorbic acid per 100 g. wet tissue.

Estimation of glycogen — Glycogen was isolated by the method of Good *et al.*[6] and estimated by the procedure of Montgomery[7] as described earlier[8].

Protein — This was estimated in whole liver homogenates by the method of Lowry *et al.*[9]. The results are expressed as mg. protein per g. wet liver. A standard solution of bovine serum albumin (fraction V) was taken in each series of estimation.

Glycogen phosphorylase (EC 2.4.1.1, α-1,4-glucan: orthophosphate glucosyl transferase) — The activity of this enzyme was assayed by the method of Shull *et al.*[10] as modified by Singh *et al.*[11]. Inorganic phosphorus was estimated by the method of Fiske and SubbaRow[12] as described earlier[8]. One unit of the enzyme required the release of 1 μmole of orthophosphate from the substrate in 1 min. at 30°C. and *p*H 5·8.

Glucose-6-phosphatase (EC 3.1.3.9, D-glucose-6-phosphate phosphohydrolase) — This was assayed by the method of Swanson[13] as modified by Shull *et al.*[10], as described earlier[8]. One unit of the enzyme was defined as the amount of the enzyme required to release 1 μmole of orthophosphate from glucose-6-phosphate in 1 min. at 30°C. and *p*H 6·4.

Fructose-1,6-diphosphatase (EC 3.1.3.11, D-fructose-1,6-diphosphate-1-phosphohydrolase)—This was assayed by the method of Freedland and Harper[14], as described earlier[8]. One unit of the enzyme was defined as the amount of enzyme required to release 1 μmole of orthophosphate from fructose-1,6-diphosphate in 1 min. at 37°C. and *p*H 9·4.

Results and Discussion

A significant reduction is observed in ascorbic acid content of tissues of guinea-pigs infected with tuberculosis (Table 1) which is not due to reduced food intake. The results of the present study are in agreement with previous reports of a similar decrease in ascorbic acid in blood[3]. Ascorbic acid has been reported to be decreased in other bacterial and viral infections such as mild coryzal fever and acute pharyngitis[15,16].

The exact biochemical function of ascorbic acid is still obscure. Ascorbic acid has been implicated in the maintenance of normal intracellular material[17] collagen synthesis, and participation in oxidation-reduction systems[18]. In tuberculosis, it is possible that there is an increased demand for utilization of ascorbic acid for many reasons. Feeding tuberculous patients with large doses of the vitamin resulted in improved resistance to the disease. Myrvik and Volk[19] found the diketo oxidation products of ascorbic acid to be strongly bacteriostatic to tubercle bacilli *in vitro* and inferred that a similar effect may be responsible for the apparent inhibitory effect of ascorbic acid *in vivo* in tuberculosis. Boyden and Anderson[20] attributed another role for ascorbic acid in minimizing the metabolic injury attending the antigen-antibody reactions believed to occur shortly after infection with tubercle bacilli. A reduction was observed in the severity of tuberculin reaction[21] in tuberculous animals after administration of ascorbic acid and this lends support to the view of Boyden and Anderson[20]. Thus the reduction in ascorbic acid content of tissues in tuberculosis could be explained by the enhanced utilization of the vitamin in resistance to infection.

It is evident from the results presented in Table 2 that the glycogen content of liver in the pair-fed animals is much less than in the *ad libitum* controls. Thus part of the reduction in glycogen content of liver observed in the present study as well as in earlier studies[1] is due to the reduced food consumption of the tuberculous guinea-pigs. Protein content

TABLE 1 — ASCORBIC ACID CONTENT IN THE TISSUES OF TUBERCULOUS GUINEA-PIGS

[Guinea-pigs were injected with 0·1 mg. dry weight of tubercle bacilli $H_{37}Rv$ in 0·1 ml. normal saline intraperitoneally. Controls received 0·1 ml. normal saline. Male guinea-pigs were sacrificed and liver, spleen, kidney and heart tissues were removed, homogenized in 4 per cent trichloracetic acid and centrifuged in the cold to remove the proteins. Ascorbic acid in the supernatant was estimated as described in the text. Values (mean ± SE) are expressed in mg. ascorbic acid/100 g. wet tissue. A value for $P \leqslant 0{\cdot}05$ is considered significant. Figures in parentheses indicate the number of animals used for analysis]

Tissue	Pair-fed controls (5)	Tuberculous (5)
Liver	18·0 ± 1·5	13·4 ± 0·8
Spleen	40·3 ± 1·8	32·5 ± 0·9
Kidney	9·1 ± 0·1	6·9 ± 0·7
Heart	5·3 ± 0·3	4·1 ± 0·3

STATISTICAL ANALYSIS

	t values	*P* values
Liver	2·70	<0·05
Spleen	3·90	<0·05
Kidney	3·99	<0·05
Heart	2·83	<0·05

TABLE 2 — GLYCOGEN AND PROTEIN CONTENT IN LIVER OF *ad libitum*, PAIR-FED AND TUBERCULOUS GUINEA-PIGS

(The pair-fed animals were given the same amount of food which was consumed by the tuberculous animals. The animals were sacrificed at the end of 4 weeks. Glycogen was isolated and estimated by the method of Good *et al.*[6]. Protein was estimated by Lowry's method in whole homogenates. Results are expressed as mean ± SE. A value for $P \leqslant 0{\cdot}05$ is considered significant. Figures in parentheses refer to the number of animals in each group. The symbols denote the following: C = control; T = tuberculous; P = pair-fed control)

Experimental condition	Glycogen g./100 g. wet liver	Protein mg./g. wet liver
Control (*ad libitum*) (5)	1·00 ± 0·03	180·0 ± 8·6
Control (pair-fed) (5)	0·61 ± 0·20	170·0 ± 10·3
Tuberculous (5)	0·29 ± 0·29	166·1 ± 2·8

STATISTICAL ANALYSIS

	C and P		P and T		C and T	
	t	*P*	*t*	*P*	*t*	*P*
Glycogen	2·1	<0·05	1·5	>0·1	16·7	<0·001
Protein	1·1	>0·1	1·37	>0·1	1·55	>0·1

TABLE 3 — ENZYMES OF CARBOHYDRATE METABOLISM IN LIVER OF CONTROL, PAIR-FED AND TUBERCULOUS GUINEA-PIGS

(Guinea-pigs were infected with tubercle bacilli and were pair-fed as described under Table 1. After sacrifice, liver was excised rapidly and enzymes in liver homogenates were assayed as mentioned in the text. Results are expressed as mean ± SE. A value for $P \leq 0.05$ was considered significant. Figures in parentheses refer to the number of animals)

Enzyme assayed	Control (5)	Tuberculous (5)	Pair-fed control (5)
Glycogen phosphorylase			
Per g. wet liver	23·4 ± 1·1	10·4 ± 0·5	18·2 ± 1·1
Per g. protein	130·0 ± 5·8	62·8 ± 6·3	100·0 ± 5·9
Glucose-6-phosphatase			
Per g. wet liver	7·5 ± 0·1	5·1 ± 0·1	8·1 ± 0·1
Per g. protein	37·0 ± 2·1	28·9 ± 5·8	42·0 ± 1·4
Fructose-1-6-diphosphatase			
Per g. wet liver	1·1 ± 0·1	2·6 ± 0·1	1·7 ± 0·04
Per g. protein	6·2 ± 0·5	15·6 ± 0·4	10·7 ± 0·2

STATISTICAL ANALYSIS

Between groups	C and T		T and P		P and C	
	t	P	t	P	t	P
Phosphorylase						
Per g. liver	10·8	<0·001	6·41	<0·05	3·3	<0·05
Per g. protein	7·8	<0·001	4·4	<0·05	3·65	<0·05
Glucose-6-phosphatase						
Per g. liver	21·2	<0·001	21·2	<0·001	4·2	<0·01
Per g. protein	13·1	<0·001	2·2	<0·05	2·1	<0·05
Fructose-1-6-diphosphatase						
Per g. liver	10·6	<0·001	8·2	<0·001	5·75	<0·001
Per g. protein	14·7	<0·001	3·45	<0·05	8·35	<0·001

of liver, on the other hand, is almost unaffected. Partial starvation is known to affect carbohydrate metabolism initially and protein metabolism later.

The results of experiments on the effect of pair feeding on the activities of phosphorylase, glucose-6-phosphatase and fructose-1-6-diphosphatase are given in Table 3. Phosphorylase activity is significantly reduced in the pair-fed animals as compared to the *ad libitum* group. This is understandable because there is a concomitant reduction in glycogen content. It is possible that the decreased phosphorylase activity observed may be an adaptation to the decreased glycogen content. Singh *et al.*[1] have made a similar observation in tuberculosis and they ascribe the reduced phosphorylase activity in the tuberculous guinea-pigs to the concomitant decrease in glycogen content in tuberculous liver. Thus, the decrease in phosphorylase observed in the present and earlier studies may be partly due to reduced food consumption in the tuberculous animals. Fructose-1-6-diphosphatase activity in the pair-fed animals is increased as is evident from the results given in Table 3. Here also reduced intake of food plays a part.

There is an enhanced activity of glucose-6-phosphatase as a result of pair feeding. In tuberculosis, on the other hand, there is a decrease in the activity of the enzyme in terms of per g. wet liver and per g. protein, but an increase in terms of 100 g. body weight[1]. Thus, it appears that the reduced food intake in tuberculous guinea-pigs apparently plays no role in the reduction of the enzyme activity in terms of per g. wet liver and per g. protein. Increase in the activity of glucose-6-phosphatase after fasting in the normal animals[22] is well known and this is in agreement with an increased activity of glucose-6-phosphatase observed in the present study.

In the present study, a reduced level of ascorbic acid was found in tissues of tuberculous guinea-pigs. Reduced intake of food by the infected animals is partly responsible for the reduction in glycogen content, reduced phosphorylase activity and increased fructose-1-6-diphosphatase activity. Protein content is apparently unaffected due to the reduced food intake in tuberculosis. The activity of glucose-6-phosphatase was increased in the pair-fed animals.

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RETARDATION OF CELL DIVISION BY VITAMIN C IN PHYSIOLOGICAL CONCENTRATIONS

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1. On the Mechanism of the Action of Ascorbic Acid in the Body

Although numerous studies have been carried out on the gross and microscopic changes occurring in tissues in vitamin C deficiency, the mechanisms by which the effects are produced are still largely unresolved.

A survey of the pathological changes leads to the conclusion that the primary effect of ascorbic acid deficiency is an abnormality in the intercellular material, or perhaps at the cell surface, leading to changes in the bones and small blood vessels. In cases of this vitamin deficiency, formation of cartilage and bone is stopped, and the junction of epiphysial cartilage and bone is weakened. The widespread occurrence of hemorrhages points to the failure of the cells in the vessel walls to adhere normally and retain their functions as closed tubes, impervious to erythrocytes. This is due to failure in the formation and maintenance of intercellular materials and consequent weakness of the tissue (Wolbach and Bessey, 1942). The phenomena are of considerable interest to the cellular physiologist for they point clearly to some relation between cell structure and pathology, arising from a biochemical deficiency. Some of these questions of cellular action have been covered in a review by Reid (1943).

It appeared to be of some value to investigate the influence of l-ascorbic acid on cell division, because of the likelihood of some action on the cell surface, which might alter conditions there so as to produce an effect on cell division which can be measured quantitatively by known methods (Shapiro, 1941). Results to date indicate that ascorbic acid does not play a major role in tissue respiration since vitamin C depleted tissues show little reduction of oxygen consumption, and but a small increase when the vitamin is added (Stotz and Harrer, 1937). Stark, Gordon and Christensen state (in Elvehjem and Wilson, 1939), "it must be recognized that there has been assigned to ascorbic acid no respiratory function in animal physiology." This does not exclude the possibility that vitamin C might still play a minor role in cell oxidations, at the same time that it determines the form of the histological picture.

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Wolbach and Howe (1926) confirmed the fact that in scorbutus vitamin C is the only factor necessary for producing normal intercellular material. The deposition of homogeneous bone matrix was observed to make its appearance within a day of administering vitamin C to scorbutic guinea pigs. An inadequate ascorbic acid level in the body affects the collagen of fibrous tissues, bone dentine and cartilage matrices, and all non-epithelial cement substances, including that of the vascular endothelium (Wolbach, 1937). Another line of evidence hinting at the importance of vitamin C in cell division is that adduced by Phillips *et al.* (1940 and 1941). Ascorbic acid is concerned with the production of virile sperm in the bull, and is involved in the early phases of reproductive processes in the cow. Vitamin C administered to impotent bulls led to an elevation of the vitamin C level in both semen and blood plasma, and to a corresponding improvement in the breeding potency of the bull.

2. Agents Affecting the Rate of Cell Division

The rate of cell division may be altered by a variety of operations, e.g., by influencing the metabolic activity of the cell, the viscous and other intracellular forces, the cell surface, or by operating on a combination of these factors. Agents like cyanide, which profoundly depress cell respiration, will completely inhibit the cell division of the sea urchin egg. Calcium, on the other hand, will accelerate or retard cell division depending upon its concentration, and appears to act primarily on the cell surface (Shapiro, 1941). An absence of calcium leads to a disintegration of the embryo, occasioned by a falling apart of the cells (Herbst, 1900), and a general appearance of softness. With adequate calcium, the rapidly changing form of the embryo passes through its normal configurations, whereas with excess calcium, the rate of cell division is retarded. In this manner, if an effect on cellular metabolism can be eliminated, the influence upon the cell surface may be studied by observing the effect on the rate of cell division. The mechanism may be either a physical or physico-chemical one; in the case of the sea urchin egg, it was suggested that the calcium may combine quantitatively with protein at the cell surface according to a mass-action relationship, and thus alter the spatial relationship between these molecules in the surface and so change its mechanical properties (*cf.* also Zweifach, 1940).

3. Vitamin C Content of Normal and Malignant Tissue

Since cancerous tissue represents an abnormal growth with numerous cell divisions in a localized area, it is relevant to examine the results of studies of the effect of ascorbic acid administration and determinations of ascorbic acid content in such tissues.

Yovarsky *et al.* (1934), investigating the vitamin C content of various human organs, arrived at a range of from about 0.55 mgm. per gram for adrenal tissue, to about 0.40 mgm. for heart tissue, with wide individual variations. Musulin and collaborators (1936) using a dye titration method, found 0.61 mgm. ascorbic acid per gram of Philadelphia No. 1 rat sarcoma, with very little necrosis; 0.04 mgm./gm. in a human large liver carcinoma with severe necrosis; and 0.18 mgm./gm. in a human benign uterine tumor with slight necrosis.

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Arloing *et al.* (1935) injected intravenously various dyes combined with vitamin C and ferrous and ferric chlorides. Occasional regressions of rabbit testicular tumors were observed, and in some human cancers of the tongue, tonsil, stomach and uterus, a retardation of growth and improvement in general condition. Severe edema was often produced near the tumor.

Boyland (1936) found that the indophenol-reducing potency of the Dael and Biltris transplantable guinea pig sarcoma is much reduced when the animal is maintained on a scorbutic diet. In this respect it behaves like normal tissues. When injected into scorbutic guinea pigs, ascorbic acid is selectively absorbed by the tumor tissue as well as by those tissues normally containing ascorbic acid. Ascorbic acid was found by Andervont and Shimkin (1939) to prevent the appearance of hemhorrage and ensuing regression of transplanted tumors treated with bacterial filtrate. They postulated that the bacterial filtrate lowered the ascorbic acid content of the tumor, and weakened its capillaries, with resultant hemorrhagic extravasation. Minor and Ramirez (1942) working with hospital patients, reported that the daily utilization of vitamin C averaged 67 mgm. for noncancerous patients, 68 mgm. for a patient with localized cancer, and 125 mgm. for patients with metastatic cancer. They suggested an accelerated usage of vitamin C by carcinomatous tissue. A. F. Watson (1936) had earlier found that the vitamin C reserves in the tissues of guinea pigs on a scorbutic diet are exhausted more rapidly if the animals are supporting rapidly growing tumors. Carruthers and Suntzeff (1942) observed that the ascorbic acid content calculated on the basis of weight of original tissue is significantly less in carcinomas than in the epidermis of normal mice, benzene-treated controls, or methylcholanthrene-treated mice. Although they found further that the ratio of ascorbic acid to nucleoprotein phosphorus was nearly the same for all groups, it does not abolish the significance of the differential ascorbic acid values found on the basis of wet weight. Robertson (1943), however, determined the ascorbic acid content of 22 tumors (of rat and mouse) and of comparable normal tissues. The ascorbic acid content of the tumors ranged from 15 to 70 mgm. per 100 gm. of fresh tissue. The ascorbic acid content of the tumors was not related to that of the tissues of origin. Robertson also concluded that no correlation between ascorbic acid concentration and rate of growth was apparent. Vogelaar and Erlichman (1937), studying the *in vitro* growth of Crocker mouse sarcoma 180, decided that vitamin C stimulates both the emigration of cells and the frequency of cell division, but furnished no quantitative measurements in substantiation.

In assessing the above results it should be borne in mind that apart from ascorbic acid, 2:6 dichloroindophenol will be non-specifically reduced by substances like stannous and ferrous salts, sulfites, sulfhydryl compounds, sulfides, thiosulfates, reductinic acid and "reductones," the latter formed by fermentation, or by splitting of sugars by heat at a suitable pH, and especially in the presence of protein (Farmer, 1944). These substances may have a structure similar to ascorbic acid with an aldol type of condensation between carbohydrate and protein derivatives.

4. Experimental Procedure

The following technic was adopted in order to measure the time required by fifty per cent of a population of cells to undergo first division in embryological development. Crystalline ascorbic acid (Eastman Kodak Co., Rochester, N. Y.)

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was used and the solutions made up shortly before use.[1] The eggs of the sea urchin, *Arabacia punctulata,* were handled as in previous studies, e.g. (Shapiro, 1941). All eggs used in any given experiment were obtained from a single urchin in order to obtain the most homogeneous cell population available. 3 ml. egg suspensions were pipetted into a pair of 300 ml. Erlenmeyer flasks, forming a shallow layer on the bottom of each flask, to facilitate gaseous interchange. The flasks were immersed in a constant temperature bath at 26.1° C. ± 0.002° C., controlled by a thermionic relay. Thermostatic control is necessary since the rate of cell division is known to be markedly affected by temperature (Loeb and Wasteneys, 1911; Hoadley and Brill, 1937). After temperature equilibration (about ten minutes), the eggs in both control and experimental flask were fertilized at the same time by the addition to each flask of 6 drops of a previously prepared suspension of sperm in sea water, and both flasks were shaken while

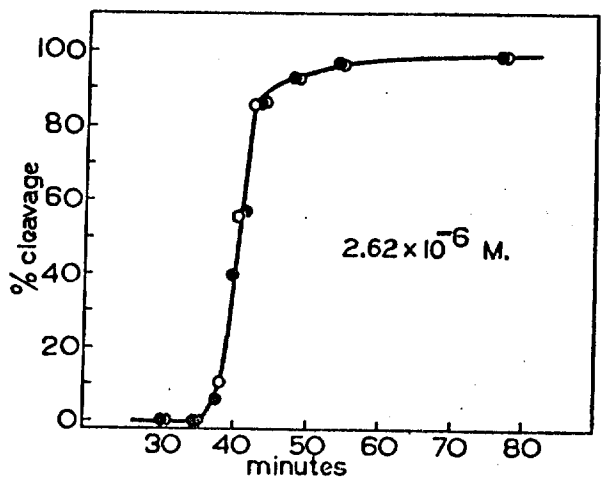

FIGURE 1. Cleavage curve showing the absence of effect at low concentrations of vitamin C, and the superposition of the two curves. Open circles, control eggs in sea water; closed circles, eggs in sea water containing 2.62×10^{-6} molar vitamin C.

immersed, to distribute the sperm. After this the flasks were clamped in a stationary position. Oxygen was passed through sea water, and then over the shallow layer of eggs in each flask, to insure adequate oxygenation throughout the experiment. At regular intervals samples of the eggs were removed, and fixed for subsequent counts of per cent cleavage (cf. Fig. 1). At much later intervals, after the experiment, the eggs were examined to check for normal developments.

Where small effects on cleavage rate are encountered, it is essential to observe three precautions outlined above, in order to obtain reproducible results. Temperature must be controlled, adequate oxygenation insured, and the curve of cleavage

[1] Owing to the buffering capacity of sea water and the small amounts of vitamin C used, it is unlikely that the pH of the sea water was reduced from its normal value (approximately 8.1) to the region where pH is known to exert an influence on cell division (below pH 5.8, Smith and Clowes, 1924).

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as a function of time determined by an adequate number of samples, and plotted. Simple observation of eggs in syracuse watch glasses at the side of the aquarium may be satisfactory where gross retardation of many minutes is observed, but for optimal quantitative results the experience of the writer has led to the adoption of the above technic. Since a population of cells is being observed, with a statistical distribution of the time at which the cell divides, sigmoid curves of the types shown in figures one and two are obtained. The slope at the midpoint is variable, and depends upon the condition of the eggs, as well as the time in the breeding season when they are used.

5. Results

The data (Fig. 2 A–D) demonstrate clearly that vitamin C added to a suspension of eggs will delay cell division at all concentrations above a minimal one. The slowing down of cleavage is more effective the higher the concentra-

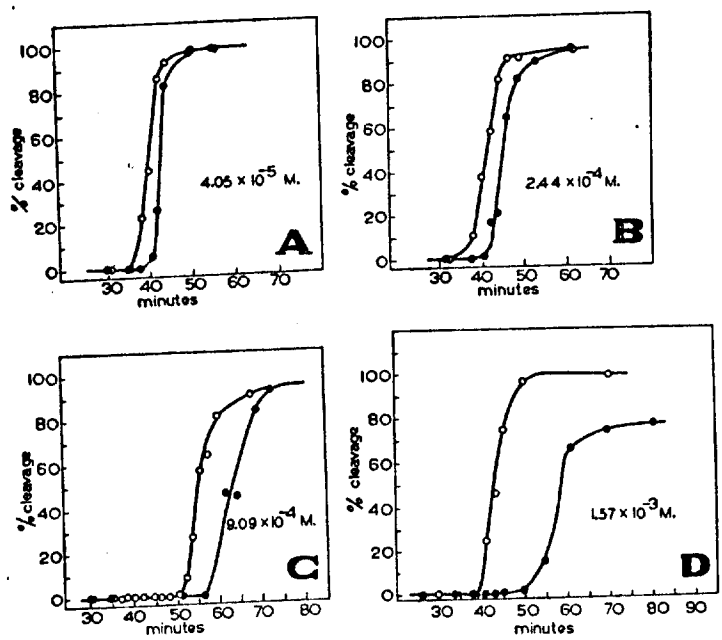

Figure 2. A, B, C and D show the increasing retardation of cell division with increasing concentration of vitamin C. The concentrations are given to the right of each pair of curves. Open circles, controls; closed circles, eggs in sea water with vitamin C.

tion, but as evidenced in Figure 2 A–C, the cells are not prevented ultimately from undergoing the same total per cent cleavage as the controls. At the concentration shown in Figure 2 D (1.57×10^{-3} molar), not only is there a considerable delay in cleavage, but it appears that in approximately 22 per cent of the cells, cleavage is entirely inhibited. At very high concentrations of vitamin C the agent

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becames toxic, and cell division can be inhibited in 100 per cent of the cells; moreover, the reaction leading to elevation of the fertilization membrane is likewise prevented. No concentration was found at which cleavage was accelerated.

In Figure 3, the amount of delay is plotted as a function of vitamin concentration. In the range studied (up to 277 micrograms per ml.), the relationship is a linear one, except at the very low concentrations, in the region of 0 to 5 micrograms per ml.

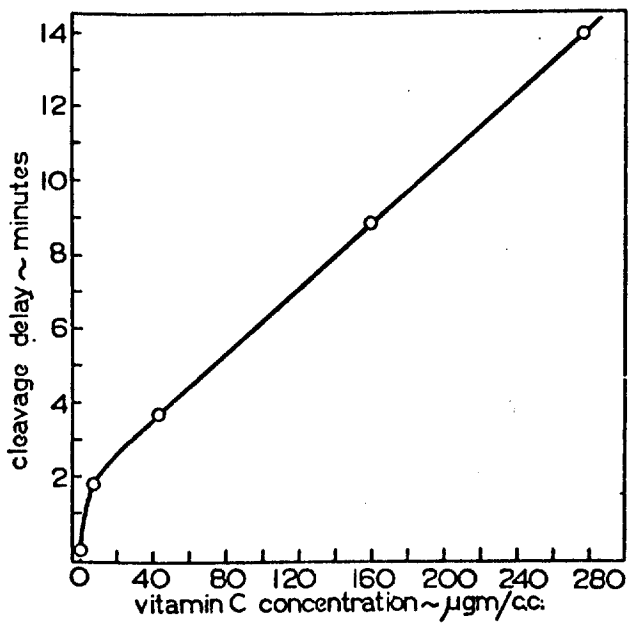

FIGURE 3. Concentration effect of vitamin C in retarding cell division.

6. SUMMARY

a. Eggs of the sea urchin, *Arbacia punctulata*, were exposed to concentrations of vitamin C in sea water, varying from 2.62×10^{-6} molar to 1.57×10^{-3} molar. The rate of cell division was determined by measuring the time required for 50 per cent of the cells to go through first cleavage.

b. Beginning with approximately 10^{-5} molar solutions, a definite retardation of cell division was observed. At high concentrations, complete inhibition of division occurred. At an intermediate concentration, the total percentage of cells dividing may be reduced from 100 per cent to some smaller figure.

c. Although cleavage was retarded in the presence of vitamin C, it appeared to be normal in other respects, with the exception of irregular cleavages at the high concentrations.

d. The slowing down of the rate of cell division appears to be roughly a linear function of the concentration, beyond a minimal concentration.

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ANDROGENICITY IN VITAMINS

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ABSTRACT

On studying androgenicity of vitamins the following results were obtained:

Vitamins E and A possess androgenic property and act synergistically with testosterone on increasing the seminal vesicle weight of castrated male rats. Vitamin B_{12} also exhibit androgenic activity but has no effect on the action of testosterone. On the other hand vitamin C though possesses no androgenic properties yet it acts synergistically with testosterone. As regards vitamin B_6, it has neither androgenic property nor synergistic action with testosterone.

The greatly increased employment of hormones in therapy attracted the investigators to search for more available sources other than the previously used hormones which are obtained from animal sources.

A surprising discovery was that vitamins possess some hormone-like properties. This finding raised the possibility of using such vitamins which are important for maintaining normal life, instead of the more expensive animal or synthetic hormones, as they can be obtained from food plants and with much less price. Since that time, many vitamins had been shown to exhibit some hormone-like effects in various degrees of potency. Also the possibility that vitamins may influence the action of hormones, which are the main internal secretions controlling body function e.g. metabolism, growth, reproduction and other vital processes which are all under the influence of endocrine secretions; suggested a study of the relationship between vitamins and hormones.

It is, therefore, proposed to undertake an investigation in order to examine various vitamins from a hormonal point of view and their possible interrelationship to gonadal hormones.

In this present study, the androgenic effect of some vitamins and their relation to testosterone has been studied on the seminal vesicles of castrated rats.

Vitamins investigated in this present study are: B_{12}, E, A, B_6, and C.

EXPERIMENTAL AND RESULTS

Androgenicity of vitamins was studied in this work, on the seminal vesicle of castrated rats. Injection was made daily for 10 days. In this respect, vitamins B_{12}, E and A were proved to possess this property, while

vitamin C and B_6 does not exhibit any androgenic activities.

The relative androgenic activity as tested on the seminal vesicle is compared in Table I.

Table I

Vitamins used	Daily dose mg.	Seminal vesicle wt.		Ratio between cont. and treated
		Control g.	Treated g.	
B_{12}	10	0.086	±0.0134 0.117	1 : 1.4
	20	0.086	±0.0073 0.112	1 : 1.42
	30	0.086 ±0.0046	±0.0083 0.124	1 : 1.44
E	30	0.088	±0.0074 0.113	1 : 1.28
	50	0.088	±0.0213 0.124	1 : 1.41
	100	0.088 ±0.0102	±0.0151 0.144	1 : 1.64
A	3000 I.U.	0.035	±0.023 0.164	1 : 4.69
	6000 I.U.	0.035 ±0.0057	±0.0062 0.186	1 : 5.31
C	40	0.144 ±0.0152	0.145 ±0.0139	1 : 1.01
	60	0.144	0.146 0.0072	1 : 1.013
B_6	10	0.118	±0.0109 0.116	1 : 0.98
	20	0.118	±0.0130 0.117	1 : 0.99
	30	0.118 ±0.0424	0.119 0.00566	1 : 1.01

It is clear from the above Table that vitamin B_{12}, E and A produced a significant increase in the seminal vesicle weight of castrated male rats while vitamin C and B_6 has no effect on that organ.

Moreover the relation between these vitamins and testosterone was also studied on the seminal vesicle weight of castrated male rats. Injection was daily for 10 days. The result is recorded in Table II.

Table II

Vitamin used	Daily dose of the vitamin	Seminal vesicle weight	
		lmg. testosterone alone	lmg. testost. + vitamin used
C	40 mg. 60 mg. 80 mg.	0.897 ±0.0458	0.941±0.0074 1.223±0.1658 1.415±0.2328
B_6	20 mg. 30 mg.	0.183 ±0.025	0.186±0.0136 0.186±0.0233
B_{12}	10 mcg. 20 mcg. 30 mcg.	1.105 ±0.103	1.118±0.0185 1.120±0.003 1.143±0.0519
E	30 mg. 50 mg. 100 mg.	1.055 ±0.2269	1.460±0.1863 1.486±0.0539 1.615±0.159
A	3000 I.U. 6000 I.U.	1.251 ±0.00831	1.721±0.0995 1.745±0.0346

The above Table indicates that vitamin C, E and A produce significant increase in the action of testosterone when they were given together, while vitamin B_6 and B_{12} has no effect on the action of testosterone.

DISCUSSION

In rats, treated with vitamin B_{12}, the mean seminal vesicle weight was 0.124 g. in comparison with 0.086 g. for the control group. This finding in male rats confirms that of Gassner et al. (1950) on cockerels. Our results, moreover, showed that there is a slight but not significant synergism between vitamin B_{12} and testosterone. However, it was proved by Sta-Mrad (1955) that injection of vitamin B_{12} to impotent patients recovered sexual activity.

Also Krzanowska (1956) showed that the capability of spermatozoa to fertilization was improved in roosters injected weekly with vitamin B_{12}.

On examining vitamin E, androgenic effects as manifested by a significant increase in development of seminal vesicle was noticed. When 30,50 or 100 mg. of vitamin E were injected daily into castrated male rats for ten days a gradual increase in the seminal vesicle weight was produced; 0.114, 0.124 & 0.144g. respectively as compared with 0.086 g. for the control. The

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ratio between the control and injected group was 1 : 1.33, 1 : 1.44 and 1 : 1.7, respectively.

Androgenic activity of vitamin E as demonstrated in this study confirms the findings of other various investigators e.g. Kudryaschov (1930), Mason (1933), Adamstone & Card (1934), Zagami (1933), Escudero et al. (1942) and Arata et al. (1962) as they proved that vitamin E deficiency produced testicular atrophy and changes in the seminal vesicles and prostate, in different animals, which are test objects for the male hormone testosterone.

In addition to the androgenic properties of vitamin E, it was also shown that this vitamin increases the action of testosterone when they were given together. The average seminal vesicle weights of groups treated with 1 mg. of testosterone and 30, 50 or 100 mg. of vitamin E are 1.460, 1.486 and $\frac{\pm 0.159}{1.615}$ as compared with $\frac{\pm 0.2269}{1.055}$ of the control group receiving testosterone alone. The ratio between the control and the treated groups was 1 : 1.38, 1 : 1.47 and 1 : 1.53 respectively. This finding is in accordance with that of Adamstone (1941) who claimed that vitamin E reinforces the action of testosterone and also Caridroit (1942) who showed that vitamin E reduces the minimum effective dose of the male hormone.

In case of vitamin A, results obtained are also very interesting since they demonstrated that this vitamin is of a high significant androgenic effect. The average seminal vesicle weight of rats receiving 3000 or 6000 I.U. of vitamin A were 0.169 and 0.186 g. while that of the control was 0.035 g. The ratio between the control and treated group was 1 : 4.83 and 1 : 5.31. The deficiency symptoms demonstrated by various investigators, indicated that this vitamin affects the male sex organs. In this respect, Gross (1924), Wolbach & Howe (1925), Myra & Korenschevsky (1932), Heinrich & Zohler (1947), Maddoch, Cohen & Wolbach (1953) and Biswas & Deb (1965), studied the effect of vitamin A deficiency on the male genital system and those results agree to a great extent to those obtained in this work.

Vitamin A, not only possesses a significant androgenic activity, but also acts synergistically with testosterone in increasing the seminal vesicle of castrated rats. The average seminal vesicle weights of the group treated with 1 mg. testosterone together with 3000 I.U. or 6000 I.U. of vitamin A and the control group receiving testosterone alone was 0.721±0.0995, 0.826±0.0346 and 0.251±0.0083 respectively. The ratio between the control and treated group is 1 : 2.87, 1 : 3.29. This result is in accordance with that of Jean & Truat (1949) who proved that vitamin A deficiency interferes with synthesis and release of androgens. Moreover Boguth & Weiser (1961) proved that, in vitamin A deficient rats, testosterone propionate did not exert its normal action.

On the other hand, Vitamin C though possessing no androgenic effect, yet it acts synergistically with testosterone (intensifying its effect on the seminal vesicles). Results showed that when 40, 60, or 80 mg. of vitamin C were given together with 1 mg. testosterone to castrated male rats, the average seminal vesible weights were 0.941 ± 0.0074, 1.223 ± 0.1658 and 1.415 ± 0.2328 respectively as compared with 0.897 ± 0.0458 for the control group receiving testosterone alone i.e. the ratio between the control and treated groups is 1 : 1.1, 1 : 1.4 and 1 : 1.6 respectively.

The synergism between vitamin C and testosterone as proved in this present investigation is in accordance with that of Morgano & Sannazzari (1952) who observed that 1 mg. of testosterone propionate has a synergistic action with vitamin C. Also Grioud & Rastimamanger (1942) showed that vitamin C is essential for the secretion of testosterone from the interstitial cells of the testicles.

As regards vitamin B_6 our results indicate that it has neither androgenic effect nor synergistic action with testosterone. This result, however, does not agree with that of Goldsmith (1952) who found that in castrated rats, supplemented with deoxypyridoxine, the seminal vesicle did not respond to injections of testosterone. Also Jeng & Kawin (1962) noticed an increase in weight of the testis in rats depleted of pyridoxine.

RESUME

Recherches sur l'activité de quelques vitamines ont délivrées les résultats suivants. Les vitamines E et A possèdent une activité androgène et agissent synergétique avec le testostérone en faisant grandir le poids des vésicules séminales de rats masculins châtrés. La vitamine B_{12} fait voir aussi une activité androgène, mais aucun effet sur l'action de testostérone. D'autre part la vitamine C possède aucune qualité androgène, mais un effet synergétique avec le testostérone. Quant à la vitamine B_6, elle fait voir ni une qualité androgène, ni un effet synergétique avec le testostérone.

ZUSAMMENFASSUNG

Untersuchungen über androgene Wirkung von Vitaminen hatten folgende Ergebnisse.

Vitamin E und Vitamin A besitzen androgene Wirkung und zeigen sich synergistisch mit Testosteron bei der Vergrößerung des Gewichtes der Seminal-Vesikel kastrierter männlicher Ratten. Vitamin B_{12} zeigt ebenso androgene Aktivität, besitzt aber keinen Einfluß auf die Wirkung des Testosterons. Vitamin C besitzt keine androgenen Eigenschaften, trotzdem wirkt es synergistisch mit Testosteron. Vitamin B_6 zeigt weder androgene Eigenschaften noch synergistische Wirkung mit Testosteron.

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OESTROGENICITY IN VITAMINS

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Natural oestrogenic hormones are of wide occurrence and although oestrogenic substances have been found in mineral sources, yet none of the naturally occurring oestrogenic hormones has been detected among them.

A surprising discovery was that vitamins possess some hormone-like properties. In this respect, Szarka (1929) found that vitamin E fed or subcutaneously injected in the form of extracts induced oestrus in 19 out of 23 infant rats. Also Giedosz (1935) stated that ascorbic acid increased the activity of the ovaries, adrenals and pituitary. These findings raised the possibility of using such vitamins instead of the more expensive animal or synthetic hormones as they can be obtained with much less price. Since that time, many vitamins had been shown to exhibit some hormone-like effects in various degrees of potency which may affect reproduction and growth activities of animals and human.

Table I

Vitamins used	Daily dose mg	Uterine Weight Control g	Uterine Weight Treated g		Ratio between control and treated
C	40 60	0.181 0.181 ± 0.0412	0.199 0.191	± 0.0096 ± 0.0261	1:1.1
B_6	20 30	0.073 0.073 ± 0.0056	0.193 0.198	± 0.0213 ± 0.0529	1:2.7
B_{12}	20mcg 30mcg	0.117 0.117 ± 0.0039	0.118 0.121	± 0.0095 ± 0.0108	1:1.03
E	30 50 100	0.085 0.085 0.085 ± 0.0056	0.087 0.099 1.822	± 0.0135 ± 0.0062 ± 0.0052	1:9.6
A	3000I.U. 6000I.U.	0.181 0.181 ± 0.014	0.182 0.185	± 0.0346 ± 0.0245	1:1.02

It is therefore, proposed to undertake an investigation in order to examine various vitamins from a hormonal point of view.

In this present investigation, the oestrogenic effect of some vitamins and their relation to oestradiol has been studied.

Vitamins investigated in this present study are: C, B_6, B_{12}, E and A.

EXPERIMENTAL AND RESULTS

Oestrogenicity of vitamins was examined in this present study on ovariectomized rats of nearly the same age and weight using the uterine weight method. Injection was made daily for 7 days. In this respect, vitamin B and E possess this property. The following table compares the relative oestrogenic activities of these vitamins.

It is clear from the above table that vitamin B_6 and E produced a significant increase in the uterine weight of ovariectomized rats while vitamin C, B_{12} and A produced no effect on that organ. Moreover, the relation between these vitamins and oestradiol was also studied on uterine w[illegible] ovariectomized rats. Injection was made daily for 7 days. The re[illegible]orded in the following table.

Table II

Vitamins used	Daily dose mg	Uterine weight	
		0.1 mcg. oestradiol	0.1 mcg. oestradiol + vitamin used
	40	0.205 ± 0.033	0.258 ± 0.0189
	60	0.205	0.276 ± 0.0436
	20	0.249 ± 0.0424	0.408 ± 0.0122
	30	0.249	0.546 ± 0.0663
B_{12}	20 mcg.	0.206 ± 0.0346	0.211 ± 0.0299
	30 mcg.	0.206	0.216 ± 0.01
	50	0.218 ± 0.0274	0.339 ± 0.0091
	100	0.218	0.454 ± 0.0178
	3000I.U	0.245 ± 0.0539	0.246 ± 0.00374
	6000I.U	0.245	0.246 ± 0.0109

The above table indicates that vitamins C, B_6 and E produce significant [illegible]ase in the action of oestradiol when they were given together, while vitamin B_{12} and A have no effect on the action of oestradiol.

DISCUSSION

In rats treated with vitamin B_6 the mean uterine weight w[illegible] comparison with 0.073 g for the control group. Moreover, [illegible] possesses a synergistic action with oestradiol when they were g[illegible] as tested on the development of the uterus. The average uter[illegible] rats treated with oestradiol and vitamin B_6 (30 g) and [illegible] oestradiol alone were: 0.546 and 0.249 g respectively [illegible] obtained with vitamin B_6 explains that of Nelson & Evans [illegible] who observed that in case of pyridoxine deficiency preg[illegible] maintained by administration of both oestrone and progest[illegible] concluded that there was inadequacy in the secretion of ova[illegible] in animals deficient in pyridoxine. Also Ross & Pike (1956) [illegible] pyridoxine is an important factor in the diet during all st[illegible] cycle. However, these findings do not agree with that of Be[illegible] who noticed that oestrogenic hormone administration and ovar[illegible] no effect on vitamin B_6 deficiency.

As regards vitamin E, results showed that on administrati[illegible] 50 mg of Vitamin E, neither cornification of the vaginal cells [illegible] increase in uterine weight of ovariectomized rats was detected [illegible] hand, when the dose was increased to 100 mg a significant [illegible] uterine weight was noticed indicating its oestrogenic effect.

The average uterine weights of the groups treated with [illegible] 100 mg of vitamin E and the control group were 0.083, 0.0[illegible] 0.086 respectively.

The oestrogenic activity of vitamin E as proved in this pr[illegible] confirms the findings of Szarka (1929) who proved that vit[illegible] subcutaneously injected in the form of extracts induced oestr[illegible] rats. Also Spagnol (1931) and Alterieux et al (1945) reported t[illegible] possesses oestrogenic activity. On the other hand Euler [illegible] Bacharach & Chance (1940) reported that vitamin E has n[illegible] activity.

It was moreover, proved in this investigation that vitamin E [illegible] the action of oestradiol on uterine development when they w[illegible] together. The average uterine weights of the groups treated w[illegible] oestradiol and 50 or 100 mg of vitamin E were: 0.339 and 0.4[illegible] tively, while the control group receiving oestradiol alone was [illegible] ratio between the control and treated group was 1:1.5 and 1:2.[illegible] not in agreement with that of Negro & Scopinaro (1941) and K[illegible] (1949) who proved that vitamin E did not stimulate the [illegible] oestradiol. On the other hand, Stahler (1941) and Stahler & H[illegible] demonstrated that vitamin E increases the action of oestradiol.

In case of vitamin C although this vitamin possesses no oestrogenic act ty, yet it intensifies the action of oestradiol on uterine development wh... they were given together. The average uterine weight of the group treated with 0.1 mcg oestradiol and 60 mg of vitamin C and that receiving 0.1 mcg oestradiol alone were 0.275 and 0.205 respectively i.e. the ratio bet een the control and injected group is 1:1.3. This significant increase in uterine weight indicates that a synergistic action exists between vitamin C and oestradiol. These findings are in accordance with that of Korenchevsky & H ll (1945) and Morgano & Sannazzari (1952) who proved the synergistic act. n between vitamin C and oestradiol. Moreover, Toshio (1956) claimed that vitamin C administration promoted ovarian function with no oes' ogenic action in itself.

regards vitamin B_{12}, our results showed that it possesses no oestrogenic action and no relation to the effect of oestradiol on uterine weight of ovariectomized rats.

testing vitamin A, it produced no obvious changes in the weight of the uterus of ovariectomized rats. This result agrees with that of Costa, Jamardo & Mazzola (1941) who found that excess vitamin A does not influence the sex cycle in rats. On the other hand, Sherwood, Brend & Ro r (1936) and Masin (1950) proved that vitamin A affects the vaginal smear and extends oestrus in rats. It was also proved in our study that vitamin A possesses no relation to oestrogen as it did not act synergistically or tically with oestradiol. This result is in agreement with that of Bu ene (1941) who found that deficiency or excess vitamin A does not increase or decrease the response of castrated rats to administered oestrogen. However, Kahn & Bern (1950), Chatterjee & Deb (1964) and oth s proved that vitamin A antagonises the action of oestradiol.

SUMMARY

studying oestrogenicity of vitamins the following results were obtained. Vitamins B_6 and E possess oestrogenic properties and act synergistically with oestradiol inc sing the uterine weight of ovariectomized rats.

amin C acts synergistically with oestradiol with no oestrogenic action in itself.

amins B_{12} and A when investigated possess no oestrogenic activity and do not act synergistically or antagonistically with oestradiol.

ZUSAMMENFASSUNG

i der Untersuchung der Östrogenwirkung von Vitaminen ergaben sich folgende Ke tate:

tamin B_6 und E besitzen östrogene Eigenschaften und wirken mit Östradiol synergistisch im Hinblick auf die Zunahme des Uterusgewichts ovariektomierter Ratten.

Vitamin C wirkt synergistisch mit Östradiol, hat aber selbst keine östrogene Wirkung.

Die Untersuchungen von Vitamin B_{12} und A ergaben weder eine östrogene Aktivität noch wirkten sie synergistisch oder antagonistisch mit Östradiol.

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FURTHER STUDIES OF THE EFFECTS OF INSULIN ON THE METABOLISM OF VITAMIN C[1]

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In studying the metabolism of ascorbic acid in normal and depancreatized dogs it was found that although the animals in both groups synthesized vitamin C and their tissues were saturated with the vitamin, as measured by test doses, the urinary excretion of ascorbic acid for a 24-hour period was significantly and consistently lower in depancreatized dogs receiving insulin than in normal dogs The major factor responsible for this difference in vitamin C excretion between the normal and the diabetic dogs proved to be the daily injections of insulin necessary to control the glycosuria in the depancreatized animal. When the insulin injections were stopped in the diabetic dogs there was a prompt increase in urinary excretion of the vitamin, the total vitamin excretion closely approximating the daily vitamin excretion prior to depancreatization. Furthermore, insulin injections caused a prompt fall in plasma concentration and urinary excretion of ascorbic acid in both normal and diabetic dogs (1). The present report is concerned with an investigation of the mechanisms involved in this reaction.

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I. THE RELATIONSHIP OF THE CHANGE IN PLASMA CONCENTRATION OF ASCORBIC ACID TO THE URINARY EXCRETION OF VITAMIN C

A constant intravenous infusion containing creatinine was given to a diabetic dog. Urine was collected by means of an indwelling catheter and preserved with sulfuric acid and hydroxyquinoline (2, 3). Blood samples were taken at frequent intervals. Regular insulin was injected once the blood level of creatinine and vitamin C were constant. Vitamin C was determined in the urine by the method of Evelyn *et al.* (4). Plasma was analyzed for ascorbic acid according to the method of Mindlin and Butler (5).

The results are given in Table I and show that the reabsorption of the vitamin is unaffected in the presence of the falling plasma concentration produced by the administration of insulin. The results indicate that the fall in urinary excretion of vitamin C following the injection of insulin is secondary to a fall in the plasma concentration of the vitamin and not due to a primary effect on the renal mechanism.

TABLE I

Effect of insulin on the renal mechanism for the reabsorption of ascorbic acid in a diabetic dog

Dog No.	Status	Time	Vit. C urine	Vit. C plasma	Glucose	Clearances			Vitamin C reabsorption	
						Vit. C	Creat.	Ratio vit. C / creat.		
		min.	*mgm. %*	*mgm. %*	*mgm. %*				*mgm./min.*	*mgm./100 cc. filtrate*
211	Diabetic	0								
		11	2.65	0.66	411	23.48	56.8	0.41	0.22	0.47
		22	2.47	0.69	410	23.04	58.6	0.39	0.25	0.42
		34	2.60	0.72	408	20.55	58.8	0.33	0.28	0.47
				Insulin 20 units I.V.						
		60	1.20	0.59	373	8.36	55.3	0.15	0.28	0.50
		78	0.36	0.49	328	2.45	57.9	0.04	0.27	0.47
		92	0.23	0.45	262	1.69	51.6	0.03	0.22	0.43

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II. EFFECT OF DIFFERENT TYPES OF INSULIN AND THE RELATIONSHIP BETWEEN THE EFFECT OF INSULIN AND THE ROUTE OF ADMINISTRATION

In the majority of the experiments regular insulin was used. To rule out the question of whether impurities might be the cause of the response to insulin, crystalline insulin was given in several experiments. This produced a rapid fall in the plasma ascorbic acid concentration similar to that caused by regular insulin.

Experiments in which the insulin was given intravenously, intramuscularly and subcutaneously showed that following the intravenous administration the effects on the plasma level of the vitamin occurred within 15 to 20 minutes, with a maximum effect at about 30 minutes; when injected intramuscularly the effect began in ½ to 1 hour with a maximum effect reached in from 2 to 3 hours; and when the insulin was given subcutaneously the effect began after 1 to 2 hours, the maximum effect was reached after 4 to 5 hours, and the duration of the effect was about 6 to 7 hours.

With the insulin doses of 10 to 40 units used in the experiments, there was no correlation between the amounts of insulin and the changes in vitamin C levels. This is not surprising as it has been observed that the duration of action of insulin on carbohydrate metabolism is not proportional to the size of the dose injected but is a function of the logarithm of the dose (6).

III. THE MECHANISMS BY WHICH INSULIN MAY CAUSE THE CHANGES OBSERVED IN THE PLASMA LEVEL AND URINARY EXCRETION OF VITAMIN C

The mechanism by which insulin caused a depression in the plasma level and urinary excretion of vitamin C remained to be investigated. The most likely explanations seemed to be either (*a*) that insulin might have a direct action on ascorbic acid catalyzing its rate of oxidation or destruction; (*b*) that in the dog insulin might block the synthesis of ascorbic acid; (*c*) that the action of insulin on carbohydrate metabolism or other intermediary metabolites might involve ascorbic acid entering into and accelerating the oxidation or utilization of these metabolites in the body and in that way increasing the utilization of vitamin C; or (*d*) that insulin might cause a redistribution of ascorbic acid within the body. A series of experiments were done to study these points.

(*a*) *Action of insulin on ascorbic acid* in vitro:

4 cc. of dog plasma were diluted with an equal amount of distilled water containing 0.8 unit of insulin. This was allowed to stand at room temperature for ½ hour before precipitating with 8 cc. of 10% metaphosphoric acid. Another 4 cc. of plasma diluted with distilled water but with no insulin added was treated in a similar manner. Determinations of the vitamin C content were done on both solutions. The solution containing the insulin had 0.49 mgm. % of vitamin C and the control solution contained 0.52 mgm. % of the vitamin. Apparently insulin had no effect. In another experiment 30 cc. of whole blood was withdrawn from a normal dog and divided into three 10 cc. specimens. To the first specimen, 1 cc. of N/saline was added and the whole blood ascorbic acid concentration determined by the method of Butler and Cushman (7). To the second specimen, 1 cc. of N/saline was added and the whole specimen was then incubated at 37° for 1 hour before determination of the blood ascorbic acid. To the third specimen, 1 cc. of N/saline containing 0.6 unit of insulin was added and the specimen was incubated for 1 hour at 37° C. The concentrations of ascorbic acid of each of the three specimens were 0.6 mgm. %.

The results show that the insulin had no direct effect *in vitro* on the ascorbic acid in whole blood or plasma.

(*b*) *Action of insulin in blocking the synthesis of ascorbic acid:*

Since the fall in the plasma concentration and urinary excretion of vitamin C following the injection of insulin was always rapid, especially when the insulin was given intravenously, it seemed unlikely that such a rapid effect would be produced simply by blocking the synthesis of the vitamin. Further evidence against this hypothesis of insulin action on vitamin C metabolism was obtained by experiments done on man, who is incapable of synthesizing the vitamin, and on dogs. Several male diabetic patients served as the human subjects. The normal fasting variations in the plasma concentration of vitamin C were determined in each diabetic patient at intervals of ½ hour for a 3-hour period. After an interval of several days the effect of injecting insulin on the plasma and urine levels of the vitamin was determined. A total of 10 experiments were done on the patients. Without insulin the plasma concentration of vitamin C did not vary by more than 0.10 mgm. % over the 3-hour period.

Following the injection of insulin there was a prompt and significant fall in the plasma concentration of the vitamin. The urinary excretion of the vitamin paralleled the changes in the plasma

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TABLE II

The effect of oral glucose and of insulin on the plasma vitamin C levels of two diabetic patients

Time	Plasma vitamin C	Blood sugar	Time	Plasma vitamin C	Blood sugar
min.	*mgm. %*	*mgm. %*	*min.*	*mgm. %*	*mgm. %*
0	1.39	552	0	0.82	
33	1.37	544	33	0.90	177
Glucose—100 gm.—orally			Glucose—100 gm.—orally		
25	1.41	624	18	0.86	308
43	1.37	648	38	0.91	372
62	1.35	656	56	0.84	348
78	1.32	704	72	0.83	320
97	1.38	672	91	0.83	300
118	1.35	600	108	0.82	292

Subject	Sz.		Subject	Sp.	
Time	Plasma vitamin C	Blood glucose	Time	Plasma vitamin C	Blood glucose
min.	*mgm. %*	*mgm. %*	*min.*	*mgm. %*	*mgm. %*
0	1.24		0	1.36	450
29	1.19	394	37	1.36	424
65	1.22	378			
Insulin 12 units I.V.			Insulin 20 units I.M.		
31	1.04	298	28	1.36	378
62	1.10	240	60	1.12	296
92	1.18	220	89	1.16	275
			Insulin 6 units I.V.		
			22	1.18	186
			52	1.04	105

levels. The average fall of plasma ascorbic acid in 10 experiments was 0.22 ± 0.07 mgm. %. In Table II are given the effects of insulin on the plasma vitamin C levels of two of the diabetic patients studied. It will be also noted that glucose alone had no effect on plasma vitamin C levels.

In another experiment a normal female dog, weighing 9¼ kg., was studied before and while receiving daily injections of insulin. The study was divided into four periods. In the first period of 7 days, the dog received no insulin; in the second period of 6 days, the animal was given one dose of 5 units of insulin subcutaneously daily; in the third period of 7 days, 10 units daily; and in the fourth period of 6 days, 15 units of insulin. Blood samples were drawn every other day prior to the injection of insulin. Daily 24-hour urinary excretions of ascorbic acid were determined.

The results of this experiment are given in Table III. The 24-hour excretion and fasting plasma concentrations of vitamin C in this normal dog did not show a decrease in any of the periods, although in acute experiments this same animal responded to injections of insulin with a fall in plasma concentration. If the 24-hour excretion of ascorbic acid is any indication of the synthesis of the vitamin in the dog, then over the 24-hour period insulin did not seem to affect the synthesis of the vitamin.

TABLE III

Effect of insulin on the 24-hour urinary excretion of vitamin C in the normal dog

No. of days	Units of insulin daily	Average 24 hr. excretion vitamin C	Fasting plasma vitamin C average
		mgm.	*mgm. %*
7	none	67	0.44
6	5	70	0.44
7	10	72	0.47
6	15	71	0.48

(*c*) *Possible action of insulin in increasing the utilization of vitamin C:*

We have presented some seemingly paradoxical data: *i.e.*, injected insulin in the normal dog causes a rapid but transient fall in plasma concentration and urinary excretion of ascorbic acid; yet the 24-hour excretion of ascorbic acid in the same dog seems unaffected by the administration of insulin. This is explained by the finding that after the acute fall of urinary vitamin C following insulin injections there is not only a return to normal but an increased excretion of the vitamin over the expected normal excretion. In another series of experiments the immediate and after effects of insulin on ascorbic acid metabolism were studied. The plasma concentration of ascorbic acid and the urinary excretion were followed hourly for periods of 10 hours. Table IV shows typical experiments. Similar findings were noted in both normal and diabetic dogs and show that, although the immediate effect of insulin is to cause a fall in plasma concentration and urinary excretion of the vitamin, once the immediate effect of the insulin has worn off there is an increased excretion of ascorbic acid for several hours and then a final return again to normal. The changes in the urinary excretion were paralleled by changes in the plasma

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TABLE IV

Experiments showing the immediate and after effects of insulin on the vitamin C levels at hourly intervals

Dog No. 227 normal

Hours	0–1		1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10
Plasma vitamin C *mgm. %*	0.42	Insulin 10 units subcutaneously	0.31	0.22	0.28	0.35	0.45	0.49	0.45		0.44
Excretion vitamin C *mgm./hour*	4.1		2.2	1.3	2.0	2.8	5.9	13.4	9.6	6.4	4.5
Plasma glucose *mgm. %*	84		62	28	33		36	61	84	95	97

Dog No. 241 diabetic

Hours	0–1		1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10
Plasma vitamin C *mgm. %*	0.57	Insulin 20 units subcutaneously	0.45	0.35	0.35	0.44	0.60	0.71	0.74	0.66	0.60
Plasma glucose *mgm. %*	256		170	74	58		48		74		108

levels of the vitamin. The latter were not as striking as the urinary changes but occurred consistently in all the animals studied. Although this explains the findings in the normal dogs, it does not explain why the diabetic dog over 24-hour periods excretes less vitamin C than does the normal dog.

In order to test the hypothesis of increased utilization after injections of insulin, a series of experiments were done on rats, humans and guinea pigs. Five normal albino rats weighing 250 to 300 gms. were placed in individual metabolism cages suspended on large funnels containing a fine mesh filter. They were fed a Purina chow diet (which is devoid of ascorbic acid) and allowed water *ad lib.* 24-hour urines were collected into dark bottles containing sulfuric acid and hydroxyquinoline. The urines were analyzed for their vitamin C content. Each animal acted as its own control. The daily excretion of ascorbic acid was observed on diet alone for 7 days; then for the next 8 days insulin was injected once daily starting with 0.2 unit subcutaneously and increasing the dose by 0.1 unit each day, so that on the 8th day each animal received 0.9 unit of insulin. The animals were then observed for 7 days longer under conditions similar to the control period.

The results of this experiment are given in Table V. They show that insulin in the doses used had no effect on the 24-hour urinary excretion of ascorbic acid. It is concluded that in the normal rat, insulin does not increase the utilization of ascorbic acid.

Experiments were also done on human subjects. As previously stated man is incapable of synthesizing ascorbic acid and as the fecal excretion of the vitamin is fairly constant and minimal (8) the 24-hour urinary excretion of the vitamin affords a measure of the difference between the daily intake and the metabolism or storage of vitamin C (9). The question of storage can be controlled by saturating an individual with the vitamin and maintaining him on a large dose daily, under which conditions the difference between

TABLE V

Effect of insulin on the daily urinary excretion of vitamin C in albino rats on the Purina chow diet

Rat No.	Age	Wt.	No. of days	Average 24-hr. urinary excretion vitamin C	Insulin* units
	days	*gm.*		*mgm.*	
1	112	277	7	1.2	none
		288	8	1.3	0.2–0.9
		299	8	1.4	none
2	112	273	7	4.1	none
		273	8	2.1	0.2–0.9
		267	7	2.4	none
3	112	286	7	5.0	none
		292	8	4.6	0.2–0.9
		293	7	5.6	none
4	112	260	7	2.3	none
		270	8	2.2	0.2–0.9
		283	7	2.5	none
5	112	262	7	3.4	none
		285	8	2.0	0.2–0.9
		285	7	2.2	none

* Rats were started on 0.2 unit of insulin daily and this was increased 0.1 unit daily.

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TABLE VI

Effect of insulin on the 24-hour urinary excretion of vitamin C in a normal adult male on 200 mgm. of vitamin C daily

No. of days	Units of insulin daily	Average 24-hr. urinary excretion of vitamin C	Average fasting plasma vit. C
		mgm. *s.d.*	*mgm. %*
9	none	70 ± 11	1.21
14	10 u.—1 day 15 u.—1 day 20 u.—1 day 30 u.—3 days 35 u.—2 days 40 u.—2 days 45 u.—4 days	84 ± 18	1.12
4	none	83	1.18

the daily intake and excretion of vitamin C is a measure of the utilization.

A normal adult male, who in previous experiments had shown a fall in the plasma level of vitamin C following injections of insulin, was maintained on a diet free of vitamin C. At the start of the experiment he was given large doses of crystalline vitamin C orally (7,100 mgm. in an 8-day period) in order to assure saturation of his tissues, and then was maintained on a daily dose of 200 mgm. Twenty-four hour urine samples were collected for determination of vitamin C and the plasma levels were done three times weekly. After a suitable control period insulin was injected subcutaneously daily beginning with 10 units and increasing to 45 units daily. The observations were continued for a short period following the withdrawal of insulin.

The results of this experiment are given in Table VI. The injections of insulin did not significantly affect the 24-hour urinary excretion of ascorbic acid, which would indicate that insulin does not increase the rate of utilization of ascorbic acid in man. This fact was further demonstrated in an experiment on guinea pigs, the results of which are given in Table VII. The onset of the signs of scurvy and the severity of the lesions at autopsy were not affected when insulin was given daily to the animals on the vitamin C-free diet. The evidence cited in experiments on dogs, rats, humans and guinea pigs does not suggest that insulin produces the acute effects on vitamin C levels through an increased utilization of the vitamin resulting in its loss from the body.

(*d*) *Does insulin cause a redistribution of vitamin C in the body tissues?*

None of the theories so far discussed adequately explains the acute effects of insulin on the plasma

TABLE VII

*Effect of the daily injection of insulin in guinea pigs on a scorbutic diet**

Pig No.	Units of insulin daily	Weight *gms.*		Day of onset of severe scurvy	Day of death	Grading of autopsy findings§							
						Bony system changes				Hemorrhages			
		Onset	Final			Long bones	Teeth	Ribs	Joints	Ribs	Intest.	Joints	Muscles
1	none	310	220	14	30‡	4+	3+	2+	3+	1+	2+	2+	4+
2	none	290	303	14	23	1+	1+	2+	2+	2+	4+	3+	3+
3	none	268	330	13	28	2+	2+	2+	2+	3+	3+	3+	4+
8	none	330	253	22	30‡	4+	1+	4+	3+	3+	4+	4+	4+
4†	0.6	281	194	16	30‡	2+	4+	2+	3+	2+	1+	3+	3+
5†	0.6	288	250	15	26	2+	4+	2+	3+	1+	1+	3+	4+
6†	0.6	350	331	15	30‡	2+	4+	4+	2+	2+	1+	4+	4+
7†	0.6	282	209	15	26	3+	4+	2+	1+	2+	4+	2+	2+
9†	0.6	326	325	18	30‡	4+	1+	2+	3+	1+	1+	3+	4+

* The diet consisted of rolled oats 39%, bran 20%, skimmed milk powder (heated to 110° C) 30%, butter fat 10% and NaCl 1%. Each pig received 1 drop of Cod Liver Oil daily.

† The pigs on insulin were started on 0.2 units daily. This was increased 0.2 units daily to a dose that caused symptoms of shock and the pig was then carried on ⅔ of that amount.

‡ These pigs were sacrificed and the vitamin C content of the plasma was determined. In every instance it was zero.

§ The findings at autopsy were graded as follows: 1+ (mild changes), 2+ (moderate), 3+ (severe), 4+ (very severe). For the bony system, the findings of looseness of the teeth, fragility of bones, spontaneous fractures of the long bones, enlargement and hemorrhages of the joints and ribs, and separation of the epiphyses were graded. For hemorrhages, the number and extent of hemorrhages were graded.

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level and urinary excretion of vitamin C. It seemed to us that a satisfactory explanation of the facts might be that insulin causes a redistribution of ascorbic acid in the body: *i.e.*, that the vitamin passes from the plasma into the tissues or tissue fluids and that, as the effect of insulin wears off, the vitamin again returns to the plasma. The proof of such an hypothesis would depend on demonstrating that coincident with the fall in plasma concentration of vitamin C there occurred a rise in concentration in the tissues or in some specific tissue and that the changes in concentration would quantitatively balance one another.

Quantitatively accurate determinations of the vitamin C content of muscles and organs under the conditions necessary in these experiments were not practical. However, the methods for determining the vitamin C content of whole blood, red blood cells and the white blood cell-platelet layer of the blood as well as the plasma were satisfactory and therefore the determination of these components of the blood were done before and after the administration of insulin. Control blood samples were taken and, following the injection of insulin, samples were collected at suitable intervals depending on the mode of administration of the insulin. Eleven experiments were done and the results are shown graphically in Figure 1 and the details are given in Table VIII. The methods of determining the vitamin C concentration in the various blood fractions were those described by Butler and Cushman (7). In 6 experiments hematocrit determinations were made and the calculated and observed values for the whole blood ascorbic acid were found to agree closely.

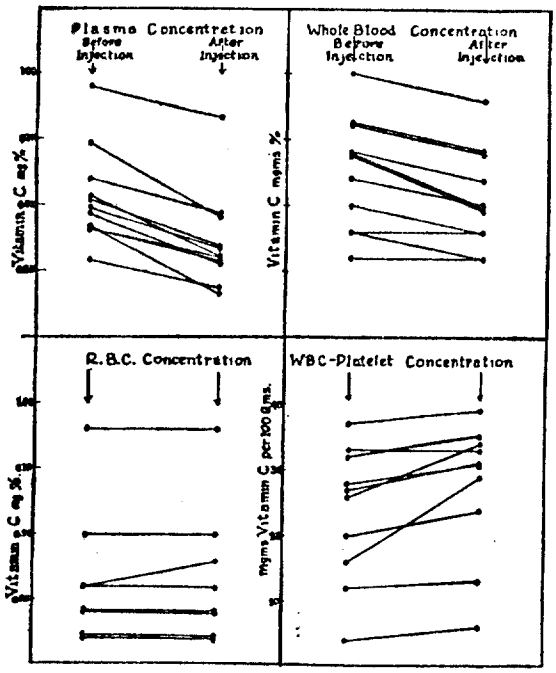

FIG. 1. CONCENTRATION OF VITAMIN C IN BLOOD CONSTITUENTS BEFORE AND AFTER INSULIN

In all of the 11 experiments there was a fall in plasma concentration of ascorbic acid after the injection of insulin. In 10 of the 11 experiments the fall can be considered significant. In one experiment the fall was within the extremes of normal variation. Of 8 experiments in which the R.B.C. concentration of ascorbic acid was determined (7 directly and one by calculation), there was no change in R.B.C. concentration of the vitamin in 7 experiments. In one experiment there was a slight rise in concentration of vitamin C following injection of insulin. In 9 of 10 experiments there was a rise in the concentration of ascorbic acid of the buffy layer (W.B.C.-platelet) after insulin; 4 of the increases were definitely significant (over 3 mgm. %), while 5 were not greater than the range of error of the method. The average determined increase after insulin in the buffy layer concentration for the 10 experiments was 4 mgm./100 gms. leukocytes. In 6 experiments calculation of the W.B.C.-platelet layer concentration of ascorbic acid from the determined hematocrit, whole blood, plasma and red blood cell concentrations showed an average rise of 5 mgm./100 gms. leukocytes following insulin administration. We think the changes are significant. There was a slight fall in whole blood concentration of vitamin C in 9 experiments. In the other two no change was noted. The results suggest that after the injection of insulin there is a fall in the plasma and the whole blood concentration of ascorbic acid; no change in the red blood cell ascorbic acid content; and a rise in the vitamin C concentration of the W.B.C.-platelet layer.

DISCUSSION

The results of the series of experiments reported here and previously (1) show that following the injection of insulin into a normal or diabetic dog or a human normal or diabetic subject, there results a fall in plasma concentration and an attendant drop in the urinary excretion of vitamin C; that this effect wears off after a variable period of time and is followed by a rise in plasma concen-

TABLE VIII

The effect of insulin on the concentration of vitamin C in the various elements of the blood

Experiment No.	Animal	Units of insulin given	Time after insulin	Vitamin C plasma	Vitamin C red blood cells	Vitamin C W.B.C.—platelet layer	Vitamin C whole blood	Blood sugar
				mgm. %	*mgm. %*	*mgm./100 gm.*	*mgm. %*	*mgm. %*
1	Dog 232	20 I.V. 15 I.V.	Fasting 1 hour	0.41 0.30	0.1 0.1	27 31	0.6 0.5	
2	Human diabetic patient	35 I.V.	Fasting 45 min.	0.95 0.83	0.9 0.9	32 35	1.0 0.9	303 123
3	Dog 234	20 Subcut.	Fasting 2 hours	0.49 0.33		26 34	0.8 0.7	
4	Dog 236	20 Subcut.	Fasting 2 hours	0.42 0.17‡		12 13	0.4 0.3	
5	Dog 234	20 Subcut.	Fasting 2 hours	0.60 0.47			0.7 0.5	Dog in Shock
6	Dog 234	20 I.V.† 20 I.M.†	Fasting 45 min.	0.53 0.31	0.2* 0.2*	33 33	0.5 0.4	98 40
7	Dog 234	20 I.V. 30 I.M.	Fasting 1 hour	0.52 0.34	0.3 0.4	37 39	0.7 0.6	
8	Dog 234	25 I.V. 25 I.M.	Fasting 1 hour	0.73 0.46	0.3 0.3	16 29	0.8 0.7	
9	Dog 227§	15 I.V. 20 I.M.	Fasting 50 min.	0.29 0.19	0.1 0.1	4 6	0.3 0.3	
10	Dog 231	20 I.V. 20 I.M.	Fasting 50 min.	0.41 0.29	0.2 0.2	20 24	0.4 0.4	
11	Dog 234	20 I.V. 10 Sub.	Fasting 45 min.	0.47 0.28	0.5 0.5	28 31	0.7 0.5	89 33

* By calculation from hematocrit.
† Crystalline insulin.
‡ Slight hemolysis present.
§ Dog had lost considerable weight and was severely diabetic.

tration and an increased excretion of ascorbic acid, and finally by a return to the normal levels. The results indicate that there is no loss of ascorbic acid from the body and suggest that vitamin C may be redistributed within the tissues coincident with some metabolic action of insulin. One would be tempted to relate this redistribution to changes in carbohydrate metabolism; but no evidence to this effect was found in the experiments done. Associated with the effect of insulin was a transfer of ascorbic acid from the plasma to the W.B.C.-platelet layer of the blood. The ability of vitamin C to shift rapidly from the plasma to the W.B.C. layer has been demonstrated both *in vitro* by Heinemann (10) and *in vivo* by ourselves in experiments on leukemic patients (11). The *in vitro* transfer of vitamin C from the W.B.C. layer to plasma is reported to be a slow process (12).

There is much indirect evidence that the W.B.C.-platelet layer concentration of ascorbic acid is an index of tissue concentration (13, 14). Since we noted a small increase in the buffy layer concentration after insulin injections, and as this by calculation only accounted for a small per cent of the temporary plasma loss of ascorbic acid and for none of the whole blood loss, it is suggested that vitamin C passes from the plasma and extracellular compartment not only into the W.B.C.-platelet layer but into the tissues as well and that it is eventually returned to the plasma. Our experiments indicate that the action does not seem to involve an actual utilization of ascorbic acid, but rather suggest that it may have some catalytic function. Haid (15) has noted a noticeable decrease in ascorbic acid content of the blood after large doses of insulin. He suggests that vitamin C wanders from the blood stream into the tissues,

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particularly the liver, and also suggests that there exists a close relationship between vitamin C and carbohydrate metabolism. Göbell and Krause (16) found that the administration of insulin and also adrenalin and nicotinamide lowered the content of reduced and dehydroascorbic acid in the blood. They suggested, as we have, that the increased metabolism in tissues temporarily deflected ascorbic acid from blood to tissues and that, after the stimulus to metabolism was over, vitamin C returned to the blood having fulfilled its role as a catalyst. If ascorbic acid does have some catalytic function in intermediary carbohydrate metabolism then one might expect to find a decreased carbohydrate tolerance in scorbutic subjects and improvement in tolerance following vitamin C therapy. This has been reported in the scorbutic guinea pig by Sigal and King (17). The literature on the relationship of ascorbic acid to carbohydrate metabolism has been confusing due to the fact that most of the observations have been on animals capable of synthesizing ascorbic acid (dogs, rabbits and rats) whose tissues are probably saturated with the vitamin, or on humans in whom the state of vitamin C nutrition has not been taken into account. (Studies of carbohydrate metabolism in human scurvy and severe ascorbic acid deficiency states, before and after vitamin C therapy, would be interesting.)

Insulin has been repeatedly demonstrated to cause a fall in plasma inorganic phosphate (18), potassium (19) and uric acid (20). It may well be that factors governing the above changes may also play a part in the changes in ascorbic acid noted.

SUMMARY

The mechanism by which insulin causes a prompt fall in plasma and urinary vitamin C levels was investigated. It was found that insulin caused these changes regardless of whether it was injected subcutaneously, intravenously or intramuscularly. There were, however, significant differences in both the onset and duration of the effects of insulin on both the plasma levels and excretion of vitamin C depending on the route of administration of the insulin. The insulin effects in each instance were transient and were followed by a period in which the plasma level and the urinary excretion were elevated. This was followed by a return of the vitamin C levels to normal.

In all species studied—man, dog and rats—these changes in vitamin C occurred in the plasma and urine following injections of insulin.

Studies in dogs, rats, guinea pigs, and humans, and determinations of the vitamin C content of blood constituents directed toward establishing the mechanism of the action of insulin on vitamin C metabolism showed: (*a*) that insulin had no effect *in vitro;* and (*b*) that insulin did not affect the synthesis of ascorbic acid. It appears that insulin causes a fall in plasma and whole blood ascorbic acid content; exerts no effect on R.B.C. ascorbic acid content; and is associated with a rise in the W.B.C.-platelet layer concentration of vitamin C.

It is suggested that insulin in its action or actions causes a transient transfer of ascorbic acid from the plasma and extracellular fluid into the tissues, possibly for some catalytic function of ascorbic acid.

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ON THE ACTION OF L-ASCORBIC ACID
I. ANTIKETOGENIC ACTION OF L-ASCORBIC ACID

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On a subject of diabetes mellitus, which was insensitive to insuline, acetone bodies were strongly proved by Legal's reaction in his urine. By the administration of insuline they did not disappear in the urine. During the period of observation, the diet was unchanged. So the writer has tried to increase carbohydrate in the diet. Glucose and L-ascorbic acid have the same carbon atoms and both are reducing substances. So it is considered that it should be possible to replace L-ascorbic acid with glucose. For this reason the writer has made the effort to find the evidence for an effect of L-ascorbic acid upon the oxidation of fatty acids. Daily 100 mg. of L-ascorbic acid were subcutaneously injected under the administration of insuline. The results were striking, and the writer recognized that the acetone bodies in urine were not only reduced but also disappeared, presumably due to the action of L-ascorbic acid.

In short, it has long been known that in the condition of fast or omission of carbohydrate from diet in man for a few days, acetone appears in breath, and acetone, acetoacetic, and β-hydroxybutyric acids are excreted in urine. Stadelmann demonstrated that the same substances were excreted in larger amounts in cases of severe diabetes and were responsible for the acidosis coming in this disease.

The precursors of the acetone bodies are known to be chiefly fats and some of the amino acids of protein, such as phenylalanine and tyrosine. The fact, first pointed out by Hirschfeld (*1*) in 1895, and well confirmed later, that they appeared when the amount of carbohydrate catabolized was small, may demonstrate a relationship between carbohydrate metabolism and the production or the non-production of the acetone bodies. Shaffer (*2*) showed that glucose and related substances such as fructose and glycerol exerted a marked effect upon the oxidation of acetoacetic acid by hydrogen peroxide in alkaline solution. At room temperature or in the incubator, and in

the absence of glucose, alkali salts of acetoacetic acid are only very slowly oxidized by hydrogen peroxide, but if glucose is present the disappearance of the keto-acid is rapid, the rate of which being increased with the rise of temperature, the increasing alkalinity of the solution and the increasing amounts of glucose. Fat is not decomposed into carbon dioxide and water, but remains in acetoacetic and β-hydroxybutyric acids—complete oxidation of fat needs simultaneously the oxidation of glucose. As an illustration, Rosenfeld (*3*) explained this relationship as follows: "Fat is burned in the fire of glucose."

Shaffer concluded that one molecule of acetoacetic acid was produced from one molecule of fatty acid, and two molecules of acetoacetic acid were completely oxidized in the presence of one molecule of glucose. This fact has been recognized as ketolytic theory of glucose for the last twenty years in the field of physiology.

In the present paper the writer will present the experiments which illustrate the ketolytic action of L-ascorbic acid, and which bring out the effect of alkalinity and temperature upon the reaction. A discussion of the reaction products and its chemical mechanism will be postponed to a later paper.

In the following paper the writer attempts to apply the conception that L-ascorbic acid will be replaced with glucose.

EXPERIMENTAL

Acetoacetic acid was prepared from the ethyl ester by saponification with 1 *N* sodium hydroxide at room temperature for 24 hours. The solution was acidified with sulfuric acid using congo red as an indicator, and thrice extracted with a half volume of ether in a separatory funnel. The ether layer was separated, shaken in the second separatory funnel with a small volume of water containing a few drops of alizarin red, and kept alkaline by the addition of NaOH. The keto-acid was quickly and completely absorbed as sodium salt by the water, and the same ether was thus used repeatedly for extraction of the saponified ester solution. The alkaline solution of the salt was aerated with a strong air current for 10 minutes in order to remove acetone, then filtered, and kept in an ice box.

When the solutions were boiled, preferably in acid solution, the keto-acid was rapidly and quantitatively decomposed into acetone which could be determined in the distillate by the iodine titration.

In alkaline solution hydrogen peroxide alone oxidizes acetoacetic

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acid very slowly at ordinary temperatures. At boiling temperature, the oxidation is more rapid during distillation, but the rapid decomposition of the acid into acetone prevents great loss, because acetone is quite resistant to peroxide and quickly distills off. Oxidation during distillation may be wholly avoided by acidifying the mixture before distillation, because neither acetoacetic acid nor acetone is attacked by hydrogen peroxide, and the yield of acetone is quantitative.

The procedure usually followed in the experiments as described below is to mix the known amounts of sodium acetoacetate in slightly alkaline solution with sodium hydroxide, hydrogen peroxide, glucose, and L-ascorbic acid, some one or more of the components being omitted in various controls.

All the mixtures were diluted to the same volume (usually 40 ml.), the flasks stoppered and placed at about the same temperature for a constant time.

In these experiments the solutions were allowed to stand at room temperature or in the incubator, and aliquot portions were withdrawn from time to time, to which 2 ml. of 10 per cent acetic acid and 40 ml. of redistilled water were added and the remaining acetoacetic acid, together with a small amount of acetone formed by the slow ketone decomposition, was determined as acetone after distillation. The reaction did not take place in acid solution.

Acetone was collected into a flask to which were added 2 ml. of sodium hydroxide solution and 5 ml. of $N/200$ iodine solution. Iodform was formed from acetone with iodine. Then, to this flask 2 ml. of 25 per cent sulfuric acid were added. Residual iodine was titrated with $N/200$ $Na_2S_2O_3$ solution. Acetoacetic acid was calculated as acetone by the following formula:

$$\{N/200\ I_2\ (ml.) - N/200\ Na_2S_2O_3\ (ml.)\} \times 0.11 \times \frac{100}{\text{amount used}} = x\ mg./dl.$$

(1 ml. of $N/200$ $Na_2S_2O_3$ solution is titrated with 0.11 mg. of acetoacetic acid as acetone.)

RESULTS

There was little, if any, disappearance of acetoacetic acid on standing at room temperature with L-ascorbic acid (in the absence of peroxide) in either neutral or alkaline solution. In the presence of hydrogen peroxide in alkaline solution, 4.1 per cent of the acetoacetic

TABLE I

Effect of L-Ascorbic Acid and Alkali on the Oxidation of Acetoacetic Acid by Hydrogen Peroxide

Solution No.	Na aceto-acetate 0.003 *M*	L-Ascorbic acid 0.001 *M*	NaOH 0.001 *N*	H_2O_2 *M*/50	(Acetoacetic acid as acetone) decomposed in distillation		
						Difference	per cent Decomposition
	ml.	*ml.*	*ml.*	*ml.*	*mg./dl.*	*mg.*	*per cent*
I	10	10	10	0	25.6		No change
II	10	0	10	0	25.6		
III	10	0	10	10	24.5	1.1	4.2
IV	10	10	10	10	4.46	21.14	82.5

at 15°

acid was oxidized. In the presence of L-ascorbic acid 82.5 per cent of the acetoacetic acid disappeared.

TABLE II

Effect of L-Ascorbic Acid and Glucose on the Oxidation of Acetoacetic Acid by H_2O_2

Solution no.	Na aceto-acetate 0.003 *M*	L-Ascorbic acid 0.001 *M*	Glucose 0.001 *M*	Acetoacetic acid (as acetone)		
					Difference	Decomposition
	ml.	*ml.*	*ml.*	*mg./dl.*	*mg.*	*per cent*
I	10	0	0	17.49		
II	10	10	0	1.76	15.73	89.9
III	10	0	10	16.38	1.11	6.3
IV	10	5	5	4.40	13.09	74.8

at 15°

Ten ml. of an approximately 0.003 *M* sodium acetoacetate solution were placed in each of four flasks, to which were added glucose and L-ascorbic acid solutions as stated in the Tables. After 2 hours at room temperature, 2 ml. portions were diluted to 40 ml., and distilled after adding 2 ml. of 10 per cent acetic acid.

The results were as follows: Shaffer showed that acetoacetic acid more rapidly disappeared in the presence of glucose. But as shown

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in the above Table, L-ascorbic acid is more effective in respect to ketolytic action than glucose. 89.9 per cent of acetoacetic acid disappears in the presence of L-ascorbic acid. In the presence of glucose the disappearance of acetoacetic acid is only 6.3 per cent. As shown in the solution no. IV in Table II, L-ascorbic acid is more powerful than the mixture of L-ascorbic acid and glucose in regards to ketolytic action.

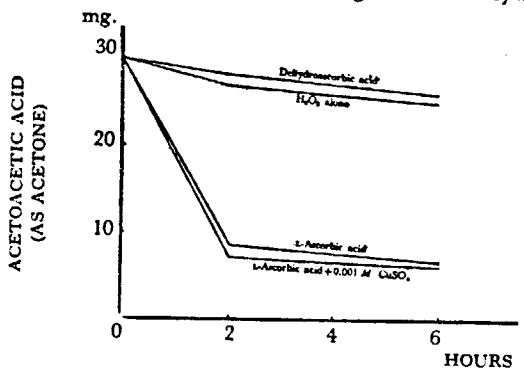

FIG. 1. Effect of L-ascorbic and dehydroascorbic acid on the oxidation of acetoacetic acid by H_2O_2

It is clear that acetoacetic acid is quickly oxidized in the presence of L-ascorbic acid as shown in Table II. The oxidation is promoted by L-ascorbic acid, but not by dehydroascorbic acid. In the presence of dehydroascorbic acid, acetoacetic acid is more slowly oxidized than control as shown in the Fig. 1. Acetoacetic acid is more rapidly oxidized in the presence of Cu^{++} (1 ml. of 0.001 *M* $CuSO_4$), but rather little in the presence of inorganic Fe^{++} (1 ml. of 0.001 *M* $FeSO_4$).

TABLE III

Effect of Alklinity on the Oxidation of Acetoacetic Acid by H_2O_2

Solution no.	Alkalinity as NaOH *N*	Acetoacetic acid as acetone		
			Difference	Decomposition
	Normal	*mg./dl.*	*mg.*	*per cent*
I	±Neutral	22.6		
II	0.0001	18.9	3.7	16.3
III	0.001	8.41	14.19	62.7
IV	0.01	7.85	14.75	65.2

at 15°

This experiment showed a marked increase in the ketolytic action of L-ascorbic acid with the increase of alkalinity of the mixture.

TABLE IV

Effect of L-Ascorbic Acid on the Oxidation of Acetoacetic Acid by H_2O_2

Solution no.	L-Ascorbic acid	Acetoacetic acid (as acetone)		
			Difference	Decomposition
	M	*mg./dl.*	*mg.*	*per cent*
I	0	26.84		
II	0.0008	20.90	5.94	22
III	0.001	4.4	22.44	83
IV	0.002	1.21	25.63	95
V	0.003	26.92	−0.08	

at 15°

As shown in Table IV, the greater is the amount of L-ascorbic acid, the faster is the disappearance of acetoacetic acid . But the oxidation is shown to be inhibited by larger amounts of L-ascorbic acid.

The results of this experiment are plotted in Fig. 2, which shows graphically the relative rate of the decomposition of acetoacetic acid under these conditions, in the presence and absence of L-ascorbic acid.

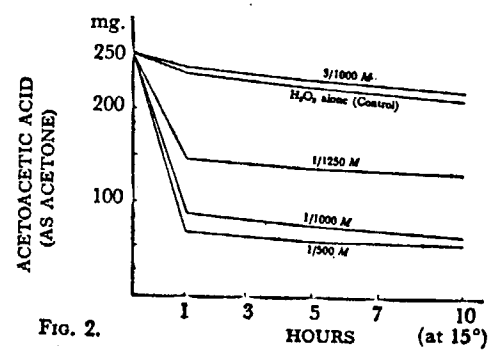

FIG. 2.

As shown in Fig. 2, the rate of ketolytic action is increased with the amount of L-ascorbic acid, but this oxidation is inhibited by larger amounts of L-ascorbic acid—that is, when the amounts of L-ascorbic acid exceed a definite molar concentration the ketolytic action does not take place, but the oxidation is later than control.

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SUMMARY

1. The oxidation of L-ascorbic acid in alkaline solution by hydrogen peroxide accomplished the disappearance of acetoacetic acid, if the latter be present in the solution. Acetoacetic acid was oxidized very slowly by hydrogen peroxide in the absence of L-ascorbic acid, but the disappearance of acetoacetic acid was rapid even at room temperature when L-ascorbic acid was simulataneously oxidized.

2. The oxidation was promoted by L-ascorbic acid, but not by dehydroascorbic acid.

3. It has been inferred that L-ascorbic acid participates the oxidation in organism.

4. The rate of ketolytic action was increased with alkalinity, as well as with the amount of L-ascorbic acid, especially in the presence of Cu^{+}. But the oxidation was inhibited when the amounts of L-ascorbic acid exceeded a definite molar concentration.

5. An illustration by Rosenfeld. "Acetoacetic acid is burned in the fire of glucose" will be applicable to this case: "Acetoacetic acid is burned in the fire of L-ascorbic acid."

Sincere thanks are due to Prof. K. Kakinuma for his criticisms and advice, and to Assistant Prof. T. Takahashi for his constant stimulating help.

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Dietary Intakes of Vitamin C in Iceland

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It has been estimated that the caloric value of the food produced in Iceland is more than three times higher than that needed to satisfy the energy requirements of the inhabitants (Sigurjonsson, 1948). More than 80% (according to caloric value) is fish and fish products, of which the greatest part is exported. The home production of milk and dairy products is sufficient except that some butter is imported and there is a surplus production of meat. The cultivation of potatoes and other vegetables, although increasing, is not yet sufficient to allow a satisfactory consumption level. There is almost no cultivation of grain so that all cereals have to be imported. Apart from some fruits (especially tomatoes) grown in hot-houses the only native fruits are berries.

It is easily understood that formerly, when potatoes and other vegetables were scarce, the diet was apt to be low in vitamin C, and probably it has been milk, more than anything else, that has saved the population from scurvy. However, scurvy was common enough, but mainly among the poorer people on the coast where milk was scarce. Until the beginning of this century the doctors frequently mentioned scurvy in their annual reports, but since then it has become more and more rare and nowadays it is only exceptionally that a clinical case of scurvy is reported.

The disappearance of scurvy has been paralleled by an increasing cultivation and consumption of potatoes and other vegetables, together with an increased consumption of milk in the towns and seaside villages. The diet, however, is still rather poor in vegetables, and fruit is scarce; one might therefore expect that the dietary intake of vitamin C is still inclined to be somewhat low.

The present survey was designed to determine how much vitamin C is available in the average national diet, the amount of this vitamin contributed by the different foodstuffs and the proportion of the total intake derived from domestic sources.

METHODS

From the annual production estimates, kindly submitted by the Director of the Agriculture Organization in Iceland, and from the official trading reports of the Statistical Bureau (Hagstofa Islands, Reykjavik, 1936–45), the average annual supply of vitamin C-containing foodstuffs for the 5-year periods 1936–40 and 1941–5 was calculated. The results are given in Table 1.

The mid-year population for 1938 was estimated as 118,290 (arithmetical mean of the census populations in 1937 and 1938, the census being taken annually at the end of the year); this is taken as the average population during the period 1936–40.

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Similarly, the mid-year population for 1943, estimated as 124,982, is taken to represent the average population for the period 1941–5.

In Table 2 the amounts of foodstuffs available are calculated in terms of average daily consumption per head of population. This table also shows the estimated amount of vitamin C thus supplied by each item, as well as the total intake derived from domestic and imported food. In addition, the second column shows how the vitamin estimates are arrived at, by giving the estimated amount of vitamin C available per

Table 1. *Total estimated yearly supplies of vitamin C-containing foodstuffs. Averages for the periods* 1936–40 *and* 1941–5, *expressed in metric tons*

Foodstuff	1936–40	1941–5
Domestic:		
Potatoes*	5,994	6,450
Swedes†	1,480	938
Carrots	18‡	26
Green vegetables	23‡	55
Tomatoes	53‡	97
Milk§	60,660	62,593
Total production consumed as such	34,930	34,620
Imported:		
Oranges	34	151
Lemons	44	85
Apples	62	494
Other fresh fruits	3	3
Dried fruits	62	773
Canned fruits	6	135
Jams, purées and syrups	39	158
Potatoes‖	941	1,432
Roots	41	5
Onions	123	175
Cabbages	80	8
Conserved vegetables (canned, dried and the like)	17	26

* The average potato crop for these two periods was estimated as 7992 and 8600 metric tons, but here 25 % has been deducted to allow for seed potatoes and storage waste.

† Crop estimates were 1850 and 1172 metric tons; 20 % has been deducted to allow for storage waste.

‡ These figures actually represent the production estimates of a single year, 1940, as no figures were available for the preceding years of this period.

§ The figures for milk consumed as such have been derived by deducting from the total for milk the amount corresponding to the estimated production of butter, cream, cheese and other milk products.

‖ 15 % has been deducted for storage waste.

100 g. of each foodstuff 'as purchased', allowance having been made for kitchen waste and cooking losses. For domestic foods the estimates are based on actual determinations carried out by the author on samples of the respective foodstuffs using the 2:6-dichlorophenolindophenol method, the reduction of the dye being determined by means of a photoelectric colorimeter. The estimates of vitamin C in the imported foods, with the exception of dried fruits, are based on the tables of the British Medical Research Council (Accessory Food Factors Committee, 1945), although slight modifications have been made for potatoes and apples. The vitamin content of dried fruits was estimated with aid of the tables of Schulerud, Kanter & Rasmussen (1945).

Table 2. *Vitamin C-containing foodstuffs; estimated vitamin C content, average daily consumption and estimated average daily intake of vitamin C/head of population*

		Average daily consumption/head			
		1936–40		1941–5	
Foodstuff	Estimated vitamin C content (mg./100 g.)	(g.)	Estimated vitamin C intake (mg.)	(g.)	Estimated vitamin C intake (mg.)
Domestic:					
Potatoes	9	138·8	12·49	141·4	12·73
Swedes	26	34·3	8·92	20·5	5·33
Carrots	4	0·4	0·02	0·6	0·02
Green vegetables	—	0·6	0·19	1·6	0·50
Tomatoes	20	1·2	0·24	2·1	0·42
Berries	—	?	?	?	?
Rhubarb	10	—	0·60	—	0·60
Syrups and jams	—	—	0·40	—	0·40
Milk consumed as such	1	809·0	8·09	758·9	7·59
Estimated intake of vitamin C from domestic foodstuffs			30·95	—	27·59
Imported:					
Oranges	41	0·8	0·33	3·3	1·35
Lemons	18	1·0	0·18	1·9	0·34
Apples	3	1·4	0·04	10·8	0·32
Other fresh fruits	—	—	0·01	—	—
Dried fruits	2	1·4	0·03	16·9	0·34
Canned fruits	5	0·1	0·01	3·0	0·15
Jams, purées and syrups	—	0·9	0·09	3·4	0·31
Potatoes	9	21·8	1·96	31·4	2·83
Roots	—	1·0	0·10	0·1	0·01
Onions	10	2·9	0·29	3·8	0·38
Cabbages	25	1·9	0·48	0·2	0·05
Conserved vegetables (canned, dried and the like)	—	0·4	0·05	3·9	0·53
Estimated intake of vitamin C from imported foodstuffs			3·57		6·61
Total estimated intake of vitamin C			34·52		34·20

Comments to Table 2

Potatoes. The estimated amount of vitamin C obtainable from 100 g. (allowance having been made for kitchen waste and cooking losses as in other similar studies) is near the average value found in autumn, but different varieties of potatoes vary to some extent in their vitamin content.

Swedes. Here also the estimated amount of vitamin C applies to autumn values.

Green vegetables. The estimate of production quoted in Table 1 is almost certainly too low. Also the amount of vitamin C obtained from 100 g. can only be estimated very roughly as this is a heterogeneous group; furthermore, some of the vegetables are eaten raw while others are cooked. In any case, however, the proportion of the total vitamin C intake obtained from this source is small.

Tomatoes. The values adopted here apply to raw tomatoes, as almost all the tomatoes are consumed raw.

Berries. Varying amounts of wild berries (*Vaccinium uliginosum*, *V. myrtilliforme* and *Empetrum nigrum*) are collected in autumn and eaten fresh. But the greater part is conserved as syrups and jams, the amounts of which are only roughly estimated, and are based on a dietary survey of some years ago.

Rhubarb. The estimate of vitamin C obtained from rhubarb also is only a very rough one.

Milk consumed as such. The vitamin C content of market milk in Reykjavik is usually about 1·0–1·5 mg./100 ml.; in the country the milk may contain about 2 mg./100 ml. when consumed fresh. As, however, a considerable amount of the milk is consumed after having been cooked, e.g. as milk soups, the average vitamin value is taken to be 1·0 mg./100 ml.

Imported foods. No allowance is made for waste during storage except for potatoes as mentioned above; the values for fresh fruits, therefore, are probably too high.

Apart from milk, some small amounts of vitamin C may be obtained from other animal foods, especially liver (from sheep and cattle) which may contain up to 20 mg./100 ml. when freshly cooked or fried. However, this source would, at best, only yield little more than 1 mg./head/day. Moreover, as fresh liver is only available in the autumn, and the greater part is conserved in various ways, no significant error will be caused by omitting this item from the table.

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RESULTS

Table 3 shows the estimated average daily total intake of vitamin C and the proportions derived from different foodstuffs.

It is striking that the average intake of vitamin C was almost exactly the same in the two periods, 1936–40 and 1941–5. In both cases the amounts derived from potatoes (home-grown and imported) and milk were much the same, these two items together

Table 3. *Estimated average daily total intake of vitamin C and proportions derived from different foodstuffs*

Period	Average total vitamin C intake (mg./head/day)	Percentage of intake derived from				
		Potatoes	Swedes	Milk	Fresh fruit	Domestic foodstuffs
1936–40	34·5	42	26	23	2	90
1941–5	34·2	45	16	22	6	81

supplying about 65% of the total intake in the first period and 67% in the second. Much fewer swedes were grown during the second period because of damage caused by the larvae of *Chortophilia brassica* especially in the southern part of the country. The effect of this on the vitamin C intake was counterbalanced by greater imports of fruits and other foodstuffs; thus in 1941–5 19% of the vitamin C intake was derived from imported food against 10% in 1936–40.

Seasonal variation

There is a considerable seasonal variation in the vitamin C intake. This is caused partly by changes in the vitamin content of some foodstuffs, chiefly potatoes, and partly by seasonal variations in the composition of the diet. From the figures obtained for the average daily intake, therefore, it cannot be inferred that the average daily intake/head of vitamin C is at a uniform level of about 34 mg. throughout the year. Green vegetables, for instance, are available only during summer and autumn and at that time potatoes contain more vitamin C than is quoted in the tables, whereas in late winter and spring the vitamin C content of potatoes may be as low as 4–5 mg./100 g. Further, the consumption of swedes, which are one of the major sources of vitamin C, is almost entirely confined to the half year from September to February, so that the amount of vitamin C derived from swedes during this period is about twice as much as indicated in Table 2. Allowing for this, it may be assumed that during autumn and early winter the average daily intake of vitamin C amounts to 40–50 mg./head/day, whereas in late winter and spring, when swedes are no longer available and potatoes are of less value, it is reduced to about 20 mg. and even lower. Now, if the average intake during spring is about 20 mg., it follows that lower values will be quite common and, allowing for a reasonable spread around the mean, it may be assumed that in not a few households the intake will be reduced to 15 mg. and, in some cases, even to about 10 mg. That this is so is supported by the results of a dietary survey carried out some years ago (Sigurjonsson, 1943). Corroborative evidence from extensive blood

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analyses is not yet available, but, from a few assays made in early spring, it seems that plasma values of about 0·1–0·2 mg. of vitamin C/100 ml., and even lower, are at least not rare.

DISCUSSION

Workers in the field of nutrition, who believe that the minimum daily requirement of vitamin C is 75–100 mg. for adults, will undoubtedly consider the results here presented as evidence of a serious shortage in vitamin C and consequently expect the population to be in a permanent state of deficiency. But what really is the minimum intake of this vitamin needed for perfect health?

Before the war the standard laid down by the League of Nations Health Organization (1938), allowing an intake of 30 mg./day for adults, was commonly accepted, at least in Europe. Later there has been a tendency to raise the standard; thus the daily allowance recommended by the U.S.A. National Research Council (1945) is 75 mg. for adults and some investigators consider the optimum intake to be about 100 mg. It is true that the term 'optimum intake' sometimes appears to be used in a different sense from that of minimum requirement intake, but really this is misleading because the lowest intake necessary to obtain optimum results should be regarded as the minimum requirement. The higher claims are based mainly on measurements of the vitamin C content of blood plasma or serum, but such measurements do not reveal any obvious level representing the limits of normality.

It has been shown by Fincke & Landquist (1942), among others, that a daily intake of well over 100 mg. of vitamin C is required to keep the blood saturated. Though few will maintain that a state of saturation is required for optimum health and nutrition, it is quite common to see the lower limit for a satisfactory plasma level put at 0·7 or 0·8 mg./100 ml. Thus Brown, Fincke, Richardson, Todhunter & Woods (1943), in grouping their material, adopt the classification of Neuweiler (1939) and accordingly take plasma values below 0·4 mg. as definitely scorbutic though not indicating clinical scurvy, values of 0·4–0·8 mg. as evidence of deficiency, and 0·8–1·0 mg. is taken by them as a good normal value. Also Fincke & Landquist (1942) seem to regard 0·8 mg./100 ml. as an index of adequate nutrition. McDevitt, Dove, Dove & Wright (1944) regard values of 0·7–1·0 mg. as satisfactory, of 0·5–0·7 mg. as suboptimal and values below 0·5 mg. as indicative of deficiency. It appears that an intake of about 60–80 mg. daily is required to keep the plasma level at 0·7–0·8 mg./100 ml. These figures for the minimum normal blood values are, however, entirely arbitrary, and it has not been shown conclusively that persons with a plasma level of, say, 0·2–0·4 mg./100 ml. or even lower, are in any way worse off than those with a level at 0·7 mg.*

* After this was written the author's attention was called to the important paper published by a committee of the British Medical Research Council on the vitamin C requirement of human adults (Vitamin C Subcommittee of the Accessory Food Factors Committee, Medical Research Council, 1948). The experiments on human volunteers there reported showed that so long as the diet did not contain more than 20 mg. of vitamin C daily, the average plasma level was below 0·1 mg./100 ml., and that at the intake level of 70 mg. daily the average plasma level was 0·55 mg./100 ml. though it seems from the accompanying graph that on some occasions it rose to above 0·7 mg./100 ml. The 'minimal protective dose', as measured by the criteria of the presence of scurvy, was found to be in the region of 10 mg. daily, though it was felt that tests of physical fatigue left some doubt whether 10 mg. was an optimal dose. It was concluded that so long as there is no evidence to support the view that an intake of more than 30 mg. daily has beneficial effects, there is no basis for recommending an intake higher than that amount.

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In the present survey it was shown that in Iceland the average intake of vitamin C/head/day during the most favourable season probably is within the range of 40–50 mg. which would correspond to a plasma level of about 0·4–0·6 mg./100 ml. This, according to the classification of Brown *et al.* (1943), as cited above, would indicate a state of deficiency even in the most favourable season and, during a great part of the year, i.e. when the average intake is from 20 to 30 mg., the blood would accordingly show 'definitely scorbutic' values. One would expect, were this true, that there would be unmistakable signs of vitamin C subnutrition in this country. This, however, is not apparent when the general state of health is considered. The infant mortality is low, usually between 30 and 40/1000 live births, the general death rate is just below 10/1000 population, and the physical development of the children may be considered good. Clinical scurvy is almost never seen, though opinions may differ about the incidence and frequency of the so-called 'latent scurvy'. Some doctors believe that this ill-defined state of hypovitaminosis is not infrequent in spring time. That this may be so appears not unlikely considering that in many single cases the intake at that time may fall below 15 mg. and probably even to about 10 mg. daily. It is commonly accepted that a daily intake of 10 mg. of vitamin C is sufficient to prevent clinical scurvy, therefore it would appear likely that, by analogy with other nutritional essentials such as minerals and protein the requirements of which are more easily determined, double that amount, i.e. about 20 mg., would be sufficient to ensure normal nutrition. It has also been shown that 20 mg. daily and even less, will cure severe cases of scurvy (Barnes, 1947; Kalk & Brühl, 1942).*

In general, the results of this survey may be taken as evidence in support of the view that 30 mg. daily of vitamin C is an adequate allowance for adults and leaves a reasonable margin of safety. Although 20 mg. may be sufficient in most cases, it is probably too low as an average intake for a group of individuals; it may be concluded, therefore, that the Icelandic diet is apt to be short in vitamin C in spring time, though this does not lead to serious consequences. The same is apparently the case in many other countries which, nevertheless, enjoy a high standard of health. Thus in Norway, according to Hagtvet (1945), the average content of vitamin C in blood serum of 722 healthy subjects fell from 0·93 mg./100 ml. in August-September to 0·16 mg. in June; in Denmark the average serum values for 6196 members of working-class families fell from 0·9 mg./100 ml. in August to 0·1 mg. in June (Andersen & Normann, 1945).

SUMMARY

1. A survey, based on estimates of production of home foods and on statistics of imported foods, showed that the average daily intake of vitamin C/head in Iceland was 34·5 and 34·2 mg. in the 5-year periods 1936–40 and 1941–5.

2. The seasonal variation was considerable, and it is shown that in the autumn and early winter the average intake was probably within the range of 40–50 mg., whereas in spring time it was about 20 mg. or less.

3. The most important sources of vitamin C in Iceland are potatoes and milk, which

* See also the footnote above, p. 279.

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together provided about two-thirds of the total intake; swedes provided, in the two periods, 26 and 15%, respectively, of the intake. The total amount of vitamin C derived from imported foodstuffs was 10% of the total intake during 1936–40 and 19% during 1941–5.

4. The population as a whole does not show obvious signs of deficiency of vitamin C, but cases of hypovitaminosis or 'subclinical scurvy' may occasionally occur in spring time. It is concluded that the standard of requirement of vitamin C accepted by the League of Nations Health Organization (1938), i.e. 30 mg. daily for adults, is fully adequate.

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Excretion of Vitamin C in Urine Following Repeated Administration of Big Test Doses

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The vitamin C saturation test as introduced by Harris & Ray (1935) is based on counting the number of days necessary for administration of standard test doses of vitamin C before a large overflow of the vitamin appears in the urine, this number increasing in proportion to the lowness of the past intake. For convenience in examining large groups of subjects, a simplified procedure, involving the collection of a single specimen of urine each day during the peak of excretion rather than of the whole day's output, was recommended by Harris & Abbasy (1937) as sufficiently accurate for routine surveys. Thus modified, the saturation test is easily applicable to group surveys and has been found capable of distinguishing between groups of children or adults at slightly different levels of intake (e.g. Harris, 1940, 1943; Atkins, 1943). The procedure adopted by various workers has, however, differed somewhat, not only as to the size of the test dose but also in choice of excretion period.

In carrying out a small-scale survey using the simplified saturation test as outlined by Harris & Abbasy (1937), it was thought that some supplementary tests, involving the estimation at graded intervals of the total 24 hr. output, might be of interest. This would give some idea of the relation in time between the peak of excretion on approaching saturation and the test period selected (e.g. a 3 hr. period, from the 4th to the 7th hour after the test dose); at the same time information might be obtained about the reliability of this short-period excretion as a criterion of the total overflow.

EXPERIMENTAL

Subjects and their treatment. Five male students at a residential college, 20–21 years of age were given big test doses of ascorbic acid by mouth on each of 4 consecutive days (6–9 June 1950).

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The test doses amounted to 10 mg./kg. body-weight and were taken in the morning except on the 1st day when they were taken at noon. Immediately before receiving the test dose the subject emptied his bladder; subsequently urine was collected at frequent intervals and analysed for vitamin C. All the subjects had undergone saturation tests about 4 months before. The dietary intakes of vitamin C were estimated to be about 20 mg./day, and serum values below 0·2 mg. vitamin C/100 ml. were found in all cases when tested just before administering the first dose.

Ascorbic-acid estimation. The ascorbic acid in the urine was estimated by the indophenol method, the reduction of the dye being determined by using a photoelectric colorimeter (Lumetron, Photovolt Corporation, New York City) instead of by titration. It was not thought necessary to correct for the blank (that is any non-specific indophenol-reducing substances).

The following procedure was used. To a measured quantity of urine (varying from 1 to 8 ml., according to a preliminary rough estimation of the concentration of the ascorbic acid) were added 2 ml. of a 20 % (w/v) solution of trichloroacetic acid, and the volume was then made up to 10 ml. with distilled water. Of the urine so diluted a suitable portion, not exceeding 2 ml., was transferred to each of a matched pair of test-tubes containing 4 ml. of an acetic acid-sodium acetate buffer of pH about 4·1. When less than 2 ml. were used, sufficient 4 % (w/v) solution of trichloroacetic acid was added to make the volume up to 6 ml. One of the tubes, after the addition of 2 ml. water, was used for zero adjustment of the colorimeter (density scale, green filter 530), and 2 ml. of a solution of dichlorophenolindophenol were then added to the other: the density reading was next taken as rapidly as possible. The difference between this reading and that of the dye control (read against water as the blank) is a measure of the extent of reduction of the dye, the dye control having been put up in the same way except that the diluted urine was replaced by an equal amount of the 4 % trichloroacetic-acid solution. The concentration of the dye solution was such as to give a reading of 5 % on the transmission scale. The dye control then gave a reading of about 2·7 on the density scale.

By this method readings can be taken rapidly, within 5–10 sec., so as to minimize the interfering action in normal urine of reducing substances other than ascorbic acid. When normal urine with low ascorbic-acid concentration (less than 1 mg./100 ml.) was tested by this method, it was found that the amount of reduction might increase considerably within the first 2 min. or so—the time often required for titration. At higher concentrations, when only small amounts of urine, diluted to one-fifth or one-tenth, were used in the test, the increase in reduction after the first reading was, however, negligible.

RESULTS

The values found for vitamin C in urine collected at intervals after the test doses are given in Table 1. Fig. 1 shows the cumulative excretion of each successive day.

On the 1st day the excretion was low in all subjects, varying from 8·7 to 19·2 mg. in the 20½ hr. Already on the 2nd day three of the subjects gave a marked response. Their response was considerably greater on the following day, but after the fourth test dose

Table 1. *Excretion of ascorbic acid in the urine of five subjects following the adminstration of one test dose (10 mg./kg. body-weight) on each of 4 consecutive days*

Period (hr. after dose)	Length of period (hr.)	H.G. (65 kg.) Urine (ml.)	H.G. Ascorbic acid mg./100 ml. urine	H.G. Ascorbic acid mg.	J.A. (80 kg.) Urine (ml.)	J.A. Ascorbic acid mg./100 ml. urine	J.A. Ascorbic acid mg.	K.S. (69 kg.) Urine (ml.)	K.S. Ascorbic acid mg./100 ml. urine	K.S. Ascorbic acid mg.	V.P. (77 kg.) Urine (ml.)	V.P. Ascorbic acid mg./100 ml. urine	V.P. Ascorbic acid mg.	G.I. (74 kg.) Urine (ml.)	G.I. Ascorbic acid mg./100 ml. urine	G.I. Ascorbic acid mg.
Before first test dose																
—	—	—	0·43		—	0·43	—		0·18			0·54		—	—	
After first test dose																
0– 4	4	584	0·57	3·33	80	3·39	2·71	194	4·64	9·00	334	1·61	5·38	250	0·71	1·78
4– 7	3	106	3·21	3·40	88	3·03	2·67	60	4·11	2·47	544	0·76	4·13	115	2·14	2·46
7–10	3	270	1·43	3·86	130	2·14	2·78	168	1·43	2·40	208	1·25	2·60	157	1·79	2·81
10–20½	10½	555	0·54	3·00	770	0·53	4·08	500	1·07	5·35	440	0·46	2·02	307	0·54	1·66
Total	20½	1515		13·59	1068		12·24	922		19·22	1526		14·13	829	—	8·71
After second test dose																
0– 2	2	180	14·3	25·7	205	1·4	2·9	85	11·4	9·7	144	1·7	2·4	167	0·9	1·5
2– 4	2	157	22·6	35·5	120	2·1	2·5	85	37·5	31·9	160	30·4	48·6	75	1·1	0·8
4– 7	3	149	6·0	8·9	169	1·9	3·2	200	13·1	26·2	220	2·9	6·4	193	0·9	1·7
7– 9	2	145	6·8	9·9	94	3·4	3·2	95	5·3	5·0	245	4·3	10·5	120	1·3	1·6
9–11	2	85	3·2	2·7	48	3·6	1·7	67	3·2	2·1	175	1·1	1·9	89	2·3	2·0
11–13	2	133	1·8	2·4	90	1·8	1·6	130	1·8	2·3	500	0·14	0·7	100	0·9	0·9
13–24	11	710	0·4	2·6	560	0·9	5·0	365	0·93	3·4	540	0·54	2·9	360	0·50	1·8
Total	24	1559	—	87·7	1286	—	20·1	1027	—	80·6	1984	—	73·4	1104	—	10·3
After third test dose																
0– 2	2	204	7·1	14·5	64	1·4	0·9	133	19·3	25·7	125	12·1	15·1	120	1·5	1·8
2– 4	2	190	42·9	81·5	120	53·6	64·3	156	89·3	139·3	172	96·4	165·8	250	12·9	32·3
4– 7	3	617	25·0	154·3	212	53·6	113·6	520	24·3	126·4	542	25·7	139·3	384	17·9	68·7
7– 9	2	170	13·6	23·1	90	8·6	7·7	146	16·9	24·7	327	4·8	15·7	115	15·7	18·1
9–11	2	205	8·6	17·6	130	8·6	11·1	158	12·9	20·4	135	3·6	4·9	223	1·0	2·2
11–13	2	222	5·7	12·7	82	4·3	3·5	202	7·9	16·0	193	7·5	14·5	120	2·1	2·5
13–23	10	685	1·1	7·5	602	2·1	12·6	460	3·5	16·1	275	3·6	9·9	655	0·43	2·8
Total	23	2293	—	311·2	1300	—	213·7	1775	—	368·6	1769	—	365·2	1867	—	128·4
After fourth test dose																
0– 2	2	} 710	28·6	203·1 {	90	19·6	17·6	585	7·9	46·2	} 80	150·0	120·0 {	120	9·3	11·2
2– 4	2				90	28·6	25·7	122	114·3	139·4				110	150·0	165·0
4– 7	3	213	78·6	167·4	190	50·0	95·0	255	35·7	91·0	370	26·1	96·6	105	152·9	160·5
7– 9	2	100	14·3	14·3	103	45·7	47·0	77	32·1	24·7	116	35·7	41·4	70	46·4	32·5

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only one of them still showed some further increase in excretion, but the other two showed a slight decline. Two of the subjects showed almost no response until after the third test dose. It was not found worth while to calculate all the values to a standard weight (70 kg.) as the main conclusions arrived at would obviously not have been thereby affected.

Table 2 shows the amounts of vitamin C excreted at certain periods on the 2nd and 3rd days, as percentages of the total output in 24 or 23 hr., respectively.

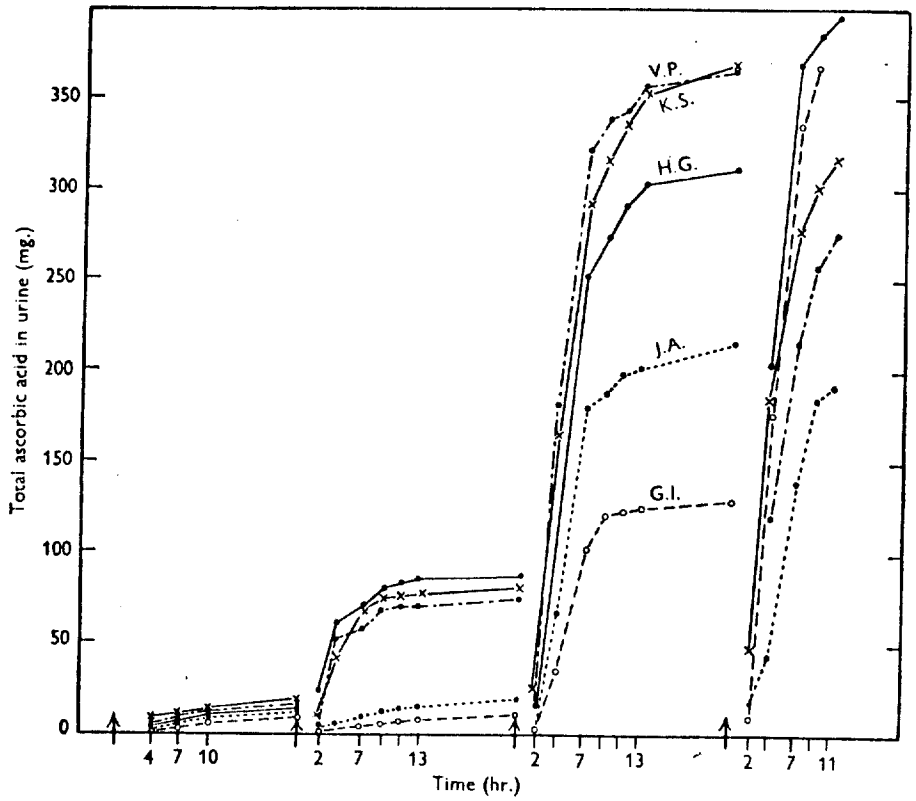

Fig. 1. Cumulative daily excretion of ascorbic acid in the urine of five male students 20–21 years old, following the administration of big test doses (10 mg./kg. body-weight) on each of 4 consecutive days, as indicated by the arrows (cf. also Table 1).

DISCUSSION

From Table 2 it is seen that when a marked response is reached (in three subjects on the 2nd day and in all on the 3rd day) a remarkably constant proportion, 94·1–97·8 %, of the 23–24 hr. output is excreted within the first 13 hr. following administration of the test dose. Also, the percentage excreted in the first 7 hr. is relatively constant, ranging from 78·2 to 87·7. With shorter periods, from the 2nd to the 4th and from the 4th to the 7th hr. after the test dose, however, this percentage varies considerably, especially during the initial response, and as often as not more was excreted in the first than in the second period. But, with one exception, both these periods together

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Table 2. *Excretion of ascorbic acid in the urine of five subjects at certain intervals after the test dose (see Table 1), expressed as a percentage of the total output in 23 or 24 hr.*

Subject	Total output: mg.	Total output: As percentage of dose	Percentage of total output excreted in interval after dose: 2–4 hr.	4–7 hr.	0–7 hr.	0–13 hr.
			2nd day, 24 hr.			
J.A.	20·1	2·5	12·4	15·9	42·8	75·1
G.I.	10·3	1·4	7·8	16·5	38·8	82·5
H.G.	87·7	13·5	40·5	10·1	79·9	97·0
K.S.	80·6	11·7	39·6	32·5	84·1	95·8
V.P.	73·4	9·5	66·2	8·7	78·2	96·0
			3rd day, 23 hr.			
J.A.	213·7	26·7	30·1	53·2	83·7	94·1
G.I.	128·4	17·4	25·2	53·5	80·1	97·8
H.G.	311·2	47·9	26·2	49·6	80·4	97·6
K.S.	368·6	53·4	37·8	34·3	79·1	95·6
V.P.	365·2	47·4	45·4	38·1	87·7	97·3

(i.e. from the 2nd to the 7th hr. after the test dose) represent a fairly constant proportion of the total output.

From this limited number of observations it would appear that the excretion during the period from 4 to 7 hr. after the test dose is not a good criterion of the magnitude of the response, as too often it does not cover the peak of excretion. Thus in two subjects (H.G. and V.P., Table 1 and Fig. 1) an initial response occurred on the 2nd day, but would hardly have been recognized if only the 4th to 7th hr. specimen had been tested. It should, however, be noted here that, although, as mentioned above, this period was originally recommended by Harris & Abbasy for the collection of the test specimen, Harris (1940, 1943) later preferred a 2¼ hr. sample taken between 3½ and 5¾ hr. after the test dose (i.e. around the 4th and 5th hr.), when he found the excretion to be at its peak.

The data contained in Table 1 relating to thirteen instances of marked response do not allow an exact timing of the peak of excretion, but obviously it has (with possibly one exception, H.G. on the 2nd day) occurred well within the range of the 2nd to the 7th hr. after the test dose and probably in all but two instances (H.G., 2nd day and J.A., 4th day) within the period from 3 to 6 hr., or even from 3 to 5½ hr., after the test dose.

Only two of the students showed continued increase in excretion up to the 4th day; the other three apparently excreted slightly less on the 4th day than on the 3rd. Unfortunately circumstances did not allow the experiment to be prolonged for some days more so as to see whether this apparent fall in excretion was of any significance. Shortly afterwards, however, similar tests were carried out with one adult male receiving a test dose of 750 mg. (10 mg./kg. body-weight) for 8 consecutive days. Fig. 2 shows the amounts of vitamin C excreted on each of these days, except the 6th when only the night specimen of urine was available. Judging from this specimen it appeared that on the 6th day the excretion was already diminishing. Anyhow, there

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was an appreciable, progressive fall for the next 2 days, in spite of continued test dosing as before. After the administration of test doses was discontinued the expected abrupt fall to a still lower level of excretion took place.

A corresponding fluctuation in the test-period excretion, in spite of continued dosage after saturation has apparently been reached, has often been observed by earlier authors (e.g. Harris, 1940, 1943). The most reasonable explanation seems to be that repeated intake of excessive amounts of ascorbic acid leads to an increased rate of destruction.

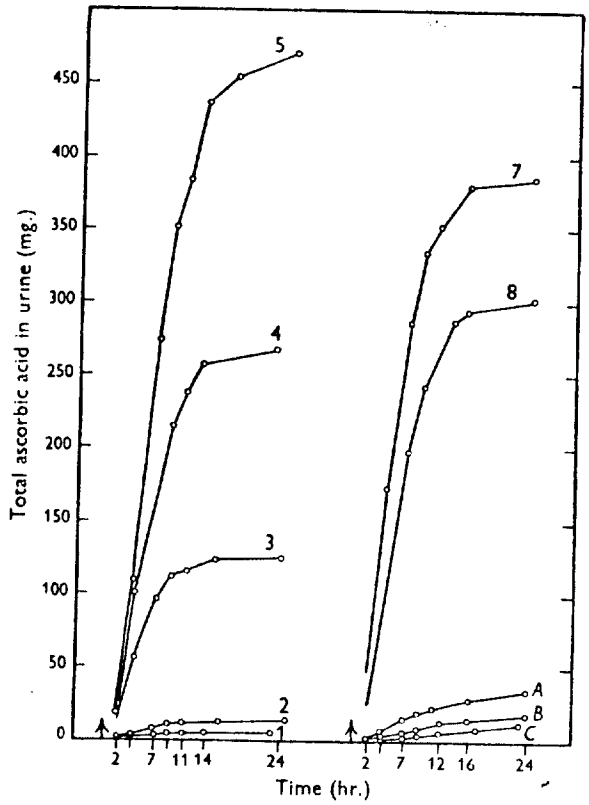

Fig. 2. Cumulative daily excretion of ascorbic acid in the urine of one adult male (weight 75 kg.) following the administration of a 750 mg. test dose on each of 8 consecutive days. (The curve for the 6th day is not shown.) The curves *A*, *B* and *C* represent the excretion on the first 3 days after discontinuing the test dosing.

The maximum output, as observed in these experiments (Tables 1 and 2, Fig. 1) when saturation is reached, is roughly about 50–60 % of the test dose, and—as far as can be judged from the 2–3 hr. afternoon samples—this seems to be in good accord with the findings of Harris (1940, 1942), and others who have used test doses of similar size. When smaller doses are given the percentage excreted may, however, be higher (Spellberg & Keeton, 1939).

Apart from losses through destruction in the tissues, it is possible that appreciable amounts of these unphysiologically large test doses are destroyed before absorption in

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the intestine. Whether or not destruction in the tissues is as great before as after saturation has been reached, it may be expected that the total losses are considerable. It therefore appears obvious that the vitamin C deficit cannot be exactly expressed quantitatively in terms of the ascorbic acid required to induce urinary response.

SUMMARY

1. Five male students were given big test doses of ascorbic acid (10 mg./kg. body-weight) on 4 consecutive days. Specimens of urine were collected at frequent intervals and analysed for ascorbic acid.

2. When a distinct response had been obtained, it was found that 94–98 % of the 23–24 hr. output of ascorbic acid were excreted in the first 13 hr., and 78–88 % in the first 7 hr. after administration of the test dose. In shorter periods within the first 7 hr. the relative amount excreted was more variable. As often as not, more was excreted in the 2 hr. period between 2 and 4 hr. after the dosage than in the following 3 hr. period. A distinct response shown by two of the subjects on the 2nd day would hardly have been noticed if the 3 hr. specimen only, collected between the 4th and 7th hr. after the test dose, had been examined.

3. Not more than about 50–60 % of the ascorbic acid administered could be recovered in the urine when full saturation was reached. This confirms previous findings that considerable losses occur through destruction or possibly through incomplete absorption of the large doses.

My thanks and appreciation are due to Mr Björn Jakobsson and Mr Bjarni Bjarnason, headmasters of the schools at Laugarvatn, for the facilities granted for the carrying out of this investigation and to the students for their excellent and conscientious co-operation.

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Ascorbic acid -a survey, past and present

A K Sim

This survey summarises investigations of the role of ascorbic acid, principally in human nutrition, carried out over the past thirty years. Beneficial effects and untoward side effects are documented and methods of analysis discussed since these have changed over this period and doubt still remains of the relevance of most recent methodology.

Role of ascorbic acid

The role of ascorbic acid in particular circumstances has been well documented[1] and the general metabolism and physiology of the vitamin has been reviewed. The latter needs only a brief mention here.

It has been recognised for some considerable time that most sources of life, with the exception of man, most primates[2] and the guinea pig, are capable of synthesising ascorbic acid. The exceptions are dependent on a regular exogenous supply of the vitamin, the optimum levels of which are described below. It is obvious from even a brief review of the literature that ascorbic acid is multifunctional and is involved in many aspects of human physiology, some of which are still poorly understood. It is involved in the tricarboxylic acid and Krebs' cycles involving body minerals, carbohydrates, fats and other vitamins. It is involved in cell function, enzyme action and protein metabolism.

Harris has classified the action of ascorbic acid in terms of:

1 Its considerable reducing activity, which may contribute to many biochemical reactions

2 Its contribution in the regeneration of tissue, collagen formation and bone deposition

3 Its participation in the conversion of folic acid to folinic acid and the metabolism of aromatic amino acids

4 Its involvement in other biological systems such as blood lipids/cholesterol levels, many of which are discussed in more detail below.

It is also considered to be intimately involved in the absorption and transport of iron from blood to tissue, in the synthesis of steroids, leucocyte activity, the function of the reticuloendothelium and in antibody formation.

Dietary requirements

The optimum daily requirements of ascorbic acid recommended by the BMA Committee on Nutrition (1950) is 20-30mg/d. In America 70mg/d is advised rising to 130mg for example during lactation.[3,4] The protective daily dose against scurvy is 10mg, although Srikantia *et al*,[5,6] have reported that this is sufficient to maintain a maximum leucocyte level in many individuals (in India) with 22mg/d or less as a maximum overall requirement.

The clinical signs of scurvy are very slow to develop and were only observed after six months on a diet containing 1mg/d ascorbic acid. For this reason, diagnostic signs of deficiency probably reflect many years of deprivation. Moreover, the author and other investigators,[7-10] have shown that there is a considerable variation in the mean leucocyte ascorbic acid level between January and May and this may even extend as far as August. Allen's report[7] also reflects this in terms of dietary intake and the availability of foods rich in the vitamin during these months. The annual average intake per person therefore may conceal an intake below the mean for up to six months of the year. In addition, it has been shown[11] that Scottish households consume more starch and less vitamin C than any other region of the UK, and current figures indicate that this imbalance is continuing.

Measurement of ascorbic acid

have been described on the macro scale, micro scale[12] and more recently, utilising automated procedures.[13]

The relevance of various measurements to the nutritional status for ascorbic acid in humans has been the subject of much controversy, and now it is generally accepted that the level of ascorbic acid in leucocytes (and platelets) of the buffy layer provides the most reliable index of vitamin C nutrition and of tissue saturation.

One of the most traditional methods for the assessment of vitamin C deficiency is the measurement of urinary excretion levels at relatively high doses of the vitamin. This 'saturation' test is fraught with difficulties, not least, that supplemented individuals excrete, in the urine, a substance which reacts chemically as ascorbic acid, but which has not yet been identified. The two compounds have been separated by thin-layer chromatography.[14]

Urinary measurements may also be complicated by a variable renal threshold or an increase in requirement and utilisation.[15] Large oral doses tend to be reflected rapidly in the plasma and subsequently in the urine without necessarily reflecting leucocyte or tissue saturation. Conversely, low-dose supplementation may be carried out over a long period with a gradual but persistent increase in leucocyte (and tissue) level without significant urinary excretion. Indeed, there are reports of individuals who appear to be impossible to saturate.[16] Burch[17] and others have shown the 'threshold' phenomenon to be clearly related to plasma ascorbic acid level while many have shown that the latter does not give a good indication of tissue saturation.[14,17–19]

It is generally agreed that one of the best methods of assessing the tissue level and nutritional status of the individual with respect to vitamin C is by measuring the level in blood leucocytes or in the leucocyte/platelet buffy layer.[5,19–21] This would seem to correlate well with the level of ascorbic acid in the tissues which is obviously of greatest significance but difficult to measure. Most leucocyte methods are adaptations of the original procedure described by Bessey and Lowry[12,22] for serum and leucocytes. In their communications, the ascorbic acid level was related to the acid-insoluble phosphorus of the sample, but more recently, this has been expressed as $\mu g/10^8$ white blood cells (WBCs) in the buffy layer.[23,24] The leucocyte count is usually carried out manually although some recent work has been reported using automated methods.[25] The measurement of ascorbic acid in the sample is based on the reaction of dehydroascorbic acid and diketogulonic acid with 2,4-dinitrophenylhydrazine.

Using this method, the leucocyte (and platelet) ascorbic acid has been shown to be directly proportional to the intake of ascorbic acid[17,26] but this is in contrast to the findings of Disselduff and Murphy.[27] This remains a major point of dispute and may not indeed be resolved until controlled trials take into account the past and present nutritional status and vitamin C level of the individual, as well as the widely reported variation in individual requirements and therefore marked variation in response to dietary supplementation.[9] A direct proportionality between leucocyte and total body ascorbic acid has been shown in the guinea pig,[17] and it is claimed that there is indirect evidence that this also holds for humans. It is not usually found to be necessary to use the conversion factor advocated by Gibson *et al*[28] in normal, thrombocytopenic, thrombocythaemic and cases of high leucocyte to platelet ratio. Cohen and Duncan[29] and others[19,27,30] have found this factor unnecessary and indeed

Alternative methods described for ascorbic acid measurement include the use of C^{14}-labelled material,[14] fluorimetric[31] and other chemical and/or colorimetric methods,[32,33] some of which have also been automated.[13,34] Very many factors may be reflected in this measurement and these must be accounted for before the relevance of the data can be appreciated. Some of the more important parameters which may influence these results are discussed in detail below.

Deficiency states

Effect of age on ascorbic acid level

The initial aim of the present survey was to examine the effect of age on leucocyte ascorbic acid level, with particular reference to those over 65 years old. It soon became obvious, however, that in the main, they were not significantly deficient and it was subsequently shown that they were not significantly different from those under 65. Instances were found of considerable deficiency among the 18-25 age group which is probably accounted for by bad dietary habits or in some instances through 'diseased' states and/or a general malabsorption. This has been confirmed by the work of Brook and Grimshaw.[25]

In general, men have been reported to be more deficient in ascorbic acid than women,[25,35–37] with a significant decline with age. This decline has not been observed in women by most workers.[38] This survey found some tendency for lower levels in elderly men although these are not significantly different from the data on females. Morgan *et al*[35] have claimed, however, that men have a higher vitamin requirement than women, but this remains very speculative.

Many reports are to be found in the literature which would appear to substantiate the theory of deficiency in the elderly.[39] Some examples of such reports are given in Table 1.

Table 1

		Leucocyte ascorbic acid level ($\mu g/10^8$ WBCs) Mean	Range
Denson and Bowers[23]	50 elderly at home (in the London area)	13·7	2-36
	Controls (working adults)	35·0	21-53
Bowers and Kubik[40]	50 elderly (mean age 76·2 years) in Industrial Midlands	12·3	2-27
Kataria *et al*[41]	25 elderly in London (at home)	26·0	
	15 elderly (hospitalised)	14·0	5-22
	16 elderly (institutionalised)	8·0	4-16

Griffiths *et al*[42] in a geriatric survey of patients admitted to hospital, indicated chemical evidence of deficiency in 41 per cent. However, in the elderly at home, the figure was 27 per cent. Brocklehurst *et al*[43] studied 80 geriatric hospital patients for one year and concluded that there was definite evidence of deficiency in many elderly people in hospital for long periods.

Andrews *et al*[44] reported a significant fall in the leucocyte ascorbic acid level in the elderly in winter. This is not surprising in view of the variation in dietary habits throughout the year. Apart from social and dietary habits and certain diseased states, it is likely that control values will depend particularly on the geographical site of the study

between 'senile purpura' and sublingual petechial haemorrhages and chronic vitamin C deficiency.[43] This, at first sight, seems very reasonable, since these clinical manifestations are well recognised in scorbutic states. Andrews and Brook,[46] however, confirmed the earlier work of Tattersall and Seville[47] which indicated that 'senile purpura' and hypovitaminosis were not related. The classical signs of deficiency, e.g. the caviare tongue (Mendes da Costa and Cremer,[48] Bean[49]) and petechial haemorrhages were indeed common in the elderly, but were shown by Andrews[9] on histological examination to be aneurysmal dilatations of the venules and mostly unrelated to the vitamin status.

Andrews and Brook[46] originally described these lesions as manifestations of deficiency, but later failed to produce any improvement by vitamin C supplementation, although they did achieve an obvious improvement in the leucocyte ascorbic acid level. There is, however, no doubt that the true petechiae of scurvy readily respond to ascorbic acid supplementation (cf Arthur *et al*[50]). Similarly, conjunctival lesions in deficiency respond rapidly to supplementation.[51]

Other authors have reported multiple vitamin deficiencies in the elderly, particularly in serum folic acid, B_{12} and ascorbic acid (Batata *et al*[52] Read *et al*[53]). These may well be interrelated and are worth evaluation in any future survey. Batata *et al*[52] have reported that while reduced food intake and malabsorption are the most likely causes of these deficiencies, they did not all respond similarly, or occur to the same degree in one individual.

Smoking

Many reports have recently indicated a possible association between cigarette smoking, related diseases and ascorbic acid deficiency.[20,54–62] Brook *et al*[25] have reported a significant reduction in plasma ascorbic acid for non-smokers with increasing age in men and women, but leucocyte levels did not change with age. Cigarette smoking, however, significantly lowered both plasma and leucocyte levels.

Indeed, it has been suggested that smoking tended to increase the 'apparent' age of an individual by 40 years with respect to leucocyte ascorbic acid level.[35] This must, however, be regarded with some suspicion and moreover serves to illustrate the difference in opinion between authors on the true effect of ageing, smoking and other parameters on different blood components. Typical figures quoted for the effect of smoking are shown in Table II.[25]

Table 2

Smoking habits	No males	Mean ascorbic acid	No females	Mean ascorbic acid
None	32	24·6 ± 1·3	50	30·7 ± 1·4
Moderate	14	19·6 ± 1·7	28	25·6 ± 1·6
Heavy	8	17·4 ± 3·5	6	27·8 ± 1·4
Moderate plus heavy	22	18·4 ± 1·6	34	26·0 ± 1·3

MacCormack[63] has claimed that smoking one cigarette destroys 25mg ascorbic acid in the body. Calder *et al*[64] have confirmed by *in vitro* tests that tobacco smoke actually destroys vitamin C in solution, but were unable to show that the plasma level was reduced in smokers or non-smokers short and long-term supplementation and depletion, that a 6h test period should indicate nothing conclusive. In most studies the reports of deficiency states associated with smoking habits fail to take into account the many other parameters which may also be involved. In other instances, there are too few subjects per group (e.g. five smokers versus five non-smokers[62]).

Bailey *et al*[65] have found no difference in the response of smokers and non-smokers in relation to ascorbic acid supplementation and exercise performance. Pelletier[54,62,66] attempted to control the diet of his patients rigorously and confirmed the results of Brook and Grimshaw.[25] By saturation tests carried out in conjunction with carefully controlled dose schedules and blood measurements, he concluded that in smokers, less ascorbic acid is effectively available for utilisation or that this group utilises the vitamin differently from non-smokers.

Ascorbic acid is involved in the synthesis of neurotransmitters such as noradrenalin and 5-hydroxytryptamine[67] and in the function of the adrenal gland. This organ contains the highest concentration of the vitamin of any tissue in the body and this must therefore have some influence on catecholamine and steroid production.

Smoking is well known to induce increased adrenalin secretion via nicotinic stimulation by nicotine and hence in smokers there is probably an abnormal demand on the adrenals and an increased utilisation of ascorbic acid at this site. Moreover, Lipscomb and Nelson[68] have shown that *in vivo*, the release of ascorbic acid precedes corticoid output with no detectable release in the absence of steroid secretion. It is also postulated by Kitabuchi[69] that a high concentration of ascorbate in the adrenal prevents steroidogenesis and therefore during stress or stimulation with ACTH (or smoking?), the adrenal must first release the bulk of its ascorbate before stimulation can commence. The effect of smoking and adrenal function could thereby represent one consistent source of ascorbic acid depletion.

It has also been shown here and elsewhere that after a prolonged period of smoking (six months or more), there is a significant proliferation of goblet cells of the epithelium of the trachea, which in turn results in an excessive secretion of mucus. Such induced cell turnover and proliferation may also represent a source of ascorbic acid depletion in smokers. The possibility of secretion of the vitamin in mucus has not been investigated, but may be of interest.

Depending on the significance of these effects, there may be a good case for the prophylactic and possibly therapeutic use of ascorbic acid in combating some of the untoward effects of heavy smoking.

Tissue damage and regeneration

While many diseased states could be classed under this heading, we are particularly concerned here with the levels of ascorbic acid in such cases as gastric/duodenal ulceration, accidental tissue damage and the effects of post-operative surgery.

Attention has recently been drawn to the low leucocyte levels of ascorbic acid in many surgical patients[70] and particularly in patients with peptic disorders.[29] The fundamental importance of vitamin C in the healing process has

Cohen and Duncan[29] found that patients with gastro-uodenal disorders had significantly lower leucocyte ascorbic acid levels than normal healthy adults. The mean levels uoted were 11·0μg/10⁸WBCs and 22·9μg/10⁸WBCs spectively. Dymock *et al*[73] confirmed these results but d that the depletion was more severe in ulcer patients nosis. Patients with previous gastric surgery and in ood health were also nearer the normal level than those milarly treated but with symptoms. They suggest, as Williamson and others[74] that this indicates inadequate bsorption or increased utilisation and not, as has been aimed, malabsorption induced by antacid administration.

Russell *et al*[20] has shown that in cases of peptic ulcer with gastrointestinal haemorrhage, the leucocyte ascorbic id is even lower and this is more exaggerated if the aemorrhage may be attributed to aspirin or alcohol induction. Similarly, Russell and Goldberg[75] reported that with pirin-induced lesions in guinea pigs, the mucosal haemorrhe which often followed, was significantly more severe hen subclinical scurvy was present. These observations ave been supported by Croft[76] who suggested that increased emorrhage in elderly people may be due to a low turnover gastric epithelial cells which rendered the mucosa partiuiarly susceptible to aspirin. The effects of aspirin in subrbutic states may also, however, be explained as a direct fect of platelet adhesiveness and the inhibition of aggreation.[77]

Typical signs of clinical scurvy have been described by illiamson *et al*[72,74] in patients who had previously underone gastric surgery and this was also associated with low vels of leucocyte ascorbic acid (12·7μg/10⁸WBCs versus 0⁸WBCs control). Collins *et al*[71] have shown a t improvement in the rate of gingival wound healing in guinea pigs and suggest that the therapeutic supplementation with ascorbic acid, at least following surgical ocedures, is indicated.

Atherosclerosis

ne of the most common features of cardiovascular disease the presence of an abnormal blood lipid pattern. This is usually manifested in raised cholesterol and triglyceride fractions, although other lipids, e.g. cholesterol esters and ospholipids are also known to be altered.

It is not yet clear what role cholesterol plays in the etiology of the disease and indeed there is considerable divided inion whether it is causitive or merely indicative of a high k situation. There is some indication that atherosclerotic plaques – certainly an important feature in thrombus formation – may be initiated by cholesterol deposition on the dothelial wall. A type of cholesterol-induced atherosclerosis cy be induced by feeding a diet rich in the sterol, but because of the short time factor involved, some doubt the evance of this lesion to the naturally-occurring intimal posit.

Sokoloff *et al*[78,79] have shown that long-term administration of ascorbic acid is effective in the prevention of is experimentally induced atherosclerosis in rats, rabbits d inea pigs, and indeed, appears to act successfully as a th ic agent in clinical atherosclerosis. Changes in iglyceride levels and lipoprotein lipase activity were served as well as a lowering of blood cholesterol. Incorporation of ascorbic acid in a high cholesterol diet in animals fectively reduced the blood lipid levels compared with rols which were only fed the cholesterol-rich diet. Similarly, the vitamin appeared to have significant protective effect in the fatty infiltrations of liver and kidney. Similar observations were reported in coronary patients with hyperlipaemic conditions.

Myasnikov[80] and others[81] have claimed that ascorbic acid markedly influenced the development of experimental atherosclerosis in the aorta of rabbits as well as alimentary cholesterolaemia. The mechanism of action proposed for the vitamin is the augmenting of ketone bodies in blood which stimulates the metabolism of fat. By increasing the functional capacity of the liver, ascorbic acid stimulates the secretion of cholesterol with bile.

The intravenous administration of ascorbic acid to patients with atherosclerosis and hypertensive disease and a high blood cholesterol level leads, according to the Soviet scientists, to a markedly lowered cholesterol and a simultaneous increase in the duodenal and faecal cholesterol levels. The antioxidant effect of the vitamin may also play an important role in fat metabolism.

In contrast, the influence of ascorbic acid deficiency in cholesterol metabolism and atherogenesis has been reported by Ginter *et al.*[82,83] This group studied the effect of chronic hypovitaminosis (C) on these parameters in cholesterol-supplemented guinea pigs and found that, while brain and blood cholesterol levels were not significantly altered in the deficient state, significantly more vascular atheromatous lesions were found in these animals. A role involving ascorbic acid in the hydroxylation of cholesterol and the formation of bile acids is proposed. Willis[84,85] has reported atheromatous changes in the aortae of scorbutic guinea pigs even without an atherogenic diet.

Chronic ascorbic acid deficiency (two weeks scorbutic regime) in guinea pigs was also reported by Ginter *et al*[82,83] to produce hypercholesterolaemia and an increased accumulation of liver cholesterol. The vitamin deficiency also increased significantly the content of saturated fatty acids (up to C_{16}) and decreased the amount of mono and polyunsaturated fatty acids and cholesterol esters of liver. In blood, the cholesterol esters contained an increased amount of linoleic acid and a decreased amount of palmitic and oleic acids. The fatty acids and triglycerides were, however, not significantly altered. A possible sex difference was also noted. These observations were thought to be due to a disturbance of cholesterol metabolism induced by the vitamin deficiency.

Shaffer[86,87] has reviewed the role of ascorbic acid in atherosclerosis and points out that local ground lesions which may be readily induced by ascorbic acid deficiency could readily be the sites of atherosclerotic/thrombotic deposits, particularly cholesterolaemic. The guinea pigs and primates, which are unable to synthesise ascorbic acid, therefore provide acceptable experimental animals for this study.

If the development of atherosclerosis via cholesterol deposition in the intima is to be accepted as relevant to human atherosclerosis, then the use of these experimental animals through the medium of induced ascorbic acid deficiency provides an ideal model system for studying the reversibility of atherosclerosis.

In a recent article Dunnigan *et al*[88] reported a seasonal variation in the incidence of myocardial infarction, which Spittle[89] has since attributed to the seasonal variation in the intake of ascorbic acid. While there may be some involvement between ascorbic acid intake, lipogenesis and cardio-

vascular disease, such sweeping claims are hardly justified.

Folic acid deficiency

Some workers believe that ascorbic acid deficiency alters folate metabolism,[7,90,91] while others claim that the dual deficiency which often exists is a result of a diet deficient in ascorbic acid and folate.[92] Folic acid and ascorbate deficiencies have been reported in several geriatric surveys[52,53,91] and while this could not be ascribed to dietary factors in all cases, it seems unlikely that the two are inter-related in any way other than in intake.

Malabsorption

Ascorbic acid deficiency is a frequent occurrence in malabsorption states, although this seldom reaches the level of clinical scurvy. Badenoch,[93] for example, has noted many instances of deficiency in steatorrhea, but only one case of clinical scurvy.

Williamson *et al*[74] have described an investigation of surgical cases (already discussed) and cases of malabsorption including pancreatic steatorrhea, reticulum cell sarcoma, intestinal diverticulosis, and idiopathic steatorrhea, all of which were significantly lower than normal in leucocyte ascorbic acid level ($10{\cdot}7\mu g/10^8$WBCs versus $20{\cdot}8\mu g/10^8$ WBCs).

Many studies of malabsorption states have indicated that the low ascorbic acid levels are not due to impaired absorption of the vitamin, but rather by way of an abnormal utilisation of requirement.[73,94] The pathogenesis of ascorbic acid malabsorption is, however, still obscure and each situation requires separate consideration.[95]

Effects of ascorbic acid

Common cold

It is generally believed that ascorbic acid supplementation may offer some protective effect against the common cold, either therapeutically or prophylactically. There is, however, very little clinical evidence to substantiate this claim and, indeed, most trials have indicated a marked beneficial effect from placebos. Under these circumstances, the claim is therefore equally difficult to disprove.[96,97]

There is evidence, however, of the therapeutic and prophylactic effects of the vitamin in certain infections,[98–104] although again it may be argued that the infection itself may be a direct result of the deficiency, or at least the latter is a contributing factor towards its development.

Ritzel[105] has claimed an improvement in upper respiratory tract infections of skiers on supplementation, while Barnes[106] has noted a reduction in the number of colds in children and basketball players with additional vitamin C.

It is tempting to suggest that, in view of the well-documented individual variation in ascorbic acid requirements, there may be a norm for each individual, above which he is less prone to infections and below which these occur more readily. Leucocytes respond rapidly to ascorbic acid supplementation and multiply equally rapidly during infection.

Mental health

Recently Pauling reiterated in the popular press the claims that ascorbic acid supplementation improves mental alertness, performance and efficiency. These claims, like the

Rheumatoid arthritis

Many years ago, Rinehart *et al*[107] reported that patients with rheumatoid arthritis were deficient in ascorbic acid. This observation has been verified since then by many workers and more recently, Abrams and Sandson[108,109] showed that the serum and synovial fluid ascorbic acid concentration in patients with rheumatoid synovitis were the same and significantly lower than in normal individuals.

Duthie[110] has confirmed the need for supplementation in rheumatoid patients and a detailed investigation of these cases has revealed essentially three groups in which the severity of the disease varies and on which ascorbic acid utilisation deficiency appears to be dependent. These groups have, as yet, not been studied in terms of their utilisation of the vitamin but are well worth further investigation.

Bone growth

The role of ascorbic acid in collagen synthesis is frequently quoted, but its direct influence on calcium mobilisation and bone growth has received less attention. The extent of the physiological function of the vitamin is unclear, but it is thought to be concerned chiefly with matrix formation.[111,112] There is evidence that the vitamin may affect the mobilisation of calcium from bone to plasma and thus it may have a more profound effect on skeletal physiology than currently believed. There is good evidence that ascorbic acid supplementation/depletion markedly affects serum alkaline phosphatase levels,[113] and this effect would explain grossly elevated enzyme levels in undernourished primates in our own colony immediately after supplementation of their diet with fresh fruit and ascorbic acid. Alkaline phosphatase levels are of course higher in immature animals, but we have noted an inexplicable rise immediately after inclusion in the colony, which gradually returns to normal when the condition of the animal is nutritionally adequate.

Miscellaneous effects

Large doses of vitamin C are said to have a beneficial effect on muscular exercise,[114] but this still remains somewhat speculative. It was believed that large quantities of the vitamin were excreted in perspiration and supplementation may be required, for example, in tropical climates.[101,102,115] Further investigation disproved this theory.

The effect of vitamin C on the rate of ethanol metabolism has been investigated[116] and appears to be without effect.

The use of ascorbic acid in conjunction with iron in anaemia has been described by many.[117–122] We have also found that the vitamin effectively increases the rate of iron absorption possibly even to the extent of dependence of dietary iron without supplementation. The response to ascorbic acid supplementation was more rapid in males than females although the effect seemed to be more long-lasting in the latter. Its effectiveness on iron absorption appears to follow a similar pattern.

Loh and Wilson[123] have recently shown a close relationship between ascorbic acid excretion and the excretion of luteinising hormone at the time of human ovulation.

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Interaction of Ascorbic Acid and Warfarin

To the Editor.—Rosenthal (215:1671, 1971) described a patient, stabilized with warfarin sodium (Coumadin), whose prothrombin time was appreciably reduced during a period of self-administration of ascorbic acid (dose not reported). Subsequently, Hume et al (219:1479, 1972) noted that 1 gm of ascorbic acid administered daily for 14 days did not interfere with the effect of warfarin on coagulability (as monitored by the Thrombotest) in five patients receiving long-term therapy with warfarin for myocardial infarction.

Our communication describes a case of unusual resistance to warfarin in a patient with acute thrombophlebitis who had been consuming approximately 16 gm of ascorbic acid per day. Laboratory studies with high doses of ascorbic acid administered orally in rabbits did not, however, reveal significant inhibition of the anticoagulant action of warfarin sodium.

Report of a Case.—A 70-year-old woman had been treated at Presbyterian-University of Pennsylvania Medical Center for acute thrombophlebitis and was discharged on a regimen of warfarin sodium, 5 mg daily. Six weeks after discharge she developed pain in the left inguinal region associated with swelling and tenderness in that area and in the lower abdomen. Twenty-four hours later, at the time of readmission, she was found to have an acute fulminating thrombophlebitis involving the left inguinal and iliac regions. The patient was febrile and in severe pain. With bed rest and active treatment of the phlebitis her course in the hospital was one of progressive improvement; however, it was unusually difficult to induce anticoagulation in this patient. Prothrombin time on admission was that of control, or 12 seconds. Dosage of warfarin sodium was increased by daily increments of 5 mg to a total of 25 mg/day before a significant increase in prothrombin time was obtained. History revealed that, on the basis of the reputed medicinal value of ascorbic acid reported in a popular magazine, she had been consuming four 500-mg tablets (assayed at 490 mg by Dr. A.R. Gennaro) of ascorbic acid on an average of eight times per day for several weeks prior to hospital readmission. The patient's subsequent course was benign and a warfarin maintenance dose of 10 mg was ultimately established. She was discharged as improved with a diagnosis of recurrent acute thrombophlebitis, left leg, and generalized arteriosclerotic cardiovascular disease. Warfarin sodium, 10 mg/day, was the only medication prescribed upon discharge.

Animal Studies.—Oral administration of ascorbic acid, 125 mg/kg (series A) and 250 mg/kg (series B) twice daily (morning and night) for five days, did not significantly affect the response to 0.1, 0.2, 0.4, and 0.8 mg/kg intravenously administered doses and an 0.8 mg/kg orally given dose of warfarin sodium in male New Zealand rabbits as determined by the one-stage prothrombin time test. These doses of ascorbic acid alone did not alter the control prothrombin time.

Comment.—As indicated in this case report, some patients may be medicating themselves with doses of ascorbic acid considerably in excess of those found by Hume and his associates, not to interfere with the anticoagulant action of warfarin. Interaction of ascorbic acid and warfarin may be a dose-related phenomenon and, although our investigations failed to demonstrate such an interaction in rabbits, additional clinical studies should be performed.

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Postulated Gastric Factor Enhancing Iron Absorption in Haemochromatosis

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SUMMARY. A variety of everted gut sac techniques have been used to determine if there is a factor in neutralized gastric juice from patients with haemochromatosis and iron deficiency which will potentiate iron absorption, as suggested by Murray (1968). No such effect was found and iron uptake by the sacs did not correlate with measurements of iron absorption in the patients with haemochromatosis using a whole body counter.

Peak iron uptake was found to occur from between 12 to 24 cm. from the rat pylorus, in the jejunum. Ascorbic acid enhanced iron transfer from the mucosal to the serosal surface but no evidence for an active transport system was found.

The role of gastric secretions in the control of iron absorption is still uncertain. Hydrochloric acid will stimulate the absorption of inorganic iron (Jacobs, Bothwell and Charlton, 1966), and in patients with an iron deficiency anaemia absorption is related to the maximal histamine induced acid output (Jacobs, Rhodes, Peters, Campbell and Eakins, 1966). However, Koepke and Stewart (1964) claimed to have found evidence for the existence of another factor in the gastric juice of anaemic dogs which would stimulate iron absorption from the intestinal tract of normal dogs, and later Murray (1965) showed that neutralized gastric juice from patients with iron deficiency anaemia or haemochromatosis would increase the uptake of labelled iron by everted rat gut segments and by the intact rat (Murray, 1968; Murray and Stein, 1968). This factor is to be distinguished from the specific chelating agent, gastroferrin, described by Davis, Luke and Deller (1966), which they suggested bound iron and thus decreased its absorption.

In this paper we describe studies using everted gut sac techniques to demonstrate the postulated gastric factor capable of stimulating iron absorption in patients with iron deficiency anaemia or haemochromatosis. The results have been correlated with simultaneous measurements of iron absorption.

MATERIALS AND METHODS

Gastric juice was obtained from 11 patients with primary idiopathic haemochromatosis, the diagnosis being made on the presence of pigmentation, hepatomegaly and usually diabetes, together with a high percentage saturation of the total iron-binding capacity, a high chelatable iron value obtained with the differential ferrioxamine test (Smith, Studley and Williams, 1967), and a liver biopsy showing the changes of cirrhosis with grade 4 iron deposition (Scheuer, Williams and Muir, 1962). The patients were examined at varying

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stages of venesection therapy. In addition, gastric juice from six patients with iron deficiency anaemia (haemoglobin 5.1–10.6 g. per 100 ml.) was assayed.

Iron absorption was measured in seven patients with haemochromatosis, using an 8 crystal whole-body counter. After a preliminary count of natural background activity, a meal of potato salad, corned beef and fruit salad, together with 0.5 μCi ^{59}Fe ferric citrate (with 0.3 μg. carrier iron) in 100 ml. water, was ingested. This meal provides approximately 10 mg. iron (Pitcher, Williams, Parsonson and Williams, 1965). The 5 hour count was taken as the 100 per cent value, and a further measurement at 14 days allowed the percentage absorption to be calculated.

Gut Segments

Young male Wistar rats, aged from 6 to 10 weeks, were starved for 16–20 hours and then killed by skull fracture. The small intestine was freed of mesentery from the pylorus to the caecum and excised *in toto*. After flushing through with ice-cold isotonic saline the intestine was cut into 3 cm. segments. These were everted over a glass rod and alternate ones were exposed to normal saline for 5 or 10 minutes as controls. The test segments were bathed in neutralized gastric juice for 5 or 10 minutes at 37° C. All segments were then incubated in conical flasks, to which oxygen had been added, in the mannose solution of Manis and Schachter (1962). 2 μCi ^{59}Fe ferrous ascorbate, with 2 μg. carrier iron, 1 ml. isotonic glucose and 0.2 ml. 10 per cent ascorbic acid were also added to the bathing fluid, and incubation proceeded at 37° C. in a shaking water bath. At the end of 1 hour the segments were removed and washed in a 0.1 per cent solution of sodium EDTA to remove any free iron by chelation. They were then dehydrated, weighed and counted in plastic tubes, using a well-type gamma scintillation counter.

Results were expressed as:

$$\text{Index of mucosal uptake of } ^{59}\text{Fe} = \frac{\text{Transferring mucosa counts/sec./g.}}{\text{Initial bathing fluid counts/sec.}}.$$

In one series of experiments gastric juice obtained after histamine stimulation was neutralized with molar 'Tris' buffer, filtered, and then concentrated by dialysis under vacuum to about half the original volume. The juice was used 24 hours after collection, whereas in all other experiments fresh juice was used.

Gut Sacs

The everted intestinal sac technique (Wilson and Wiseman, 1954) was employed, using adult male Wistar rats (250–400 g.). The method was similar to that used for gut segments except that 6 or 10 cm. lengths were cut and made into sacs filled with Ringer-Tyrode solution. The sacs were constructed in varying sequences, so that the possible effect of time lost prior to incubation was randomized. The test sacs were incubated for 1 hour at 37° C in gastric juice to which 2 μCi ^{59}Fe ferric citrate (carrier iron content 0.3 μg.) had been added. After incubation the sacs were dried with filter paper, the inner pouch fluid was drained, and that part of the sac remaining after removal of the ligated ends was placed in a desiccator for 24 hours and weighed. The radioactivity of the pouch fluid, the bathing fluid

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and the dehydrated sections was then counted. The following indices of Brown and Justus (1958) were calculated:

$$\text{Index of mucosal uptake of } {}^{59}\text{Fe} = \frac{\text{Transferring mucosa counts/sec./g.}}{\text{Initial bathing fluid counts/sec.}}$$

$$\text{Index of pouch fluid uptake of } {}^{59}\text{Fe} = \frac{\text{Pouch fluid counts/sec./g. mucosa}}{\text{Initial bathing fluid counts/sec.}} \times 100.$$

RESULTS

Gut Segments

Exposure of the segments to neutralized gastric juice from patients with either iron deficiency anaemia or haemochromatosis did not increase the uptake of ^{59}Fe. Gastric juice from patients with haemochromatosis which was concentrated by dialysis was equally ineffective (Table I).

TABLE I

THE UPTAKE OF ^{59}FE BY GUT SEGMENTS EXPOSED TO GASTRIC JUICE

Gastric juice	*No. of tests*	*Time of exposure (min.)*	*Index mucosal uptake of ^{59}Fe*	
			Control	*Test*
Haemochromatosis	4	5	14.3 (0.92)	14.7 (3.47)
Haemachromatosis	4	10	12.6 (1.14)	6.7 (1.33)
Haemochromatosis (concentrated)	3	10	8.8 (0.52)	8.0 (2.46)
Iron deficiency	6	10	12.7 (0.94)	11.5 (1.39)

Mean values are given with the standard error in parentheses.

TABLE II

COMPARISON OF EFFECTS OF GASTRIC JUICE FROM PATIENTS WITH IRON DEFICIENCY OR HAEMOCHROMATOSIS USING GUT SACS

	Index sac fluid uptake of ^{59}Fe		*Index mucosal uptake of ^{59}Fe*	
	Haemochromatosis (11)	*Iron deficiency* (6)	*Haemochromatosis* (11)	*Iron deficiency* (6)
Test	17.5 (2.20)	11.37 (3.40)	4.26 (0.70)	2.35 (0.41)
Control	11.4 (1.33)	7.6 (1.65)	3.32 (0.61)	2.29 (0.43)

Mean values shown with standard error in parentheses.

Gut Sacs

In this series of experiments 10 cm. gut sacs were used. The third and sixth sacs which were incubated in Ringer-Tyrode solution containing a similar concentration of isotope served as controls. Gastric juice from 11 subjects with haemochromatosis and from six with

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iron deficiency anaemia was tested. No striking difference was found between the control and test sacs (Table II). Mucosal and sac fluid uptake of labelled iron was a little higher in the

TABLE III

COMPARISON OF IRON ABSORPTION AND UPTAKE OF ^{59}Fe BY GUT SACS IN SEVEN PATIENTS WITH IDIOPATHIC HAEMOCHROMATOSIS

Subject	*Mean index mucosal uptake of ^{59}Fe*	*Mean index sac fluid uptake of ^{59}Fe*	*Iron absorption (%)*
F.T.	3.41	6.9	41
G.B.	4.81	32.7	11
A.G.	2.03	14.3	19
J.W.	1.33	5.9	97
F.G.	3.28	12.1	7
J.K.	3.21	18.1	10
P.B.	1.34	17.5	75

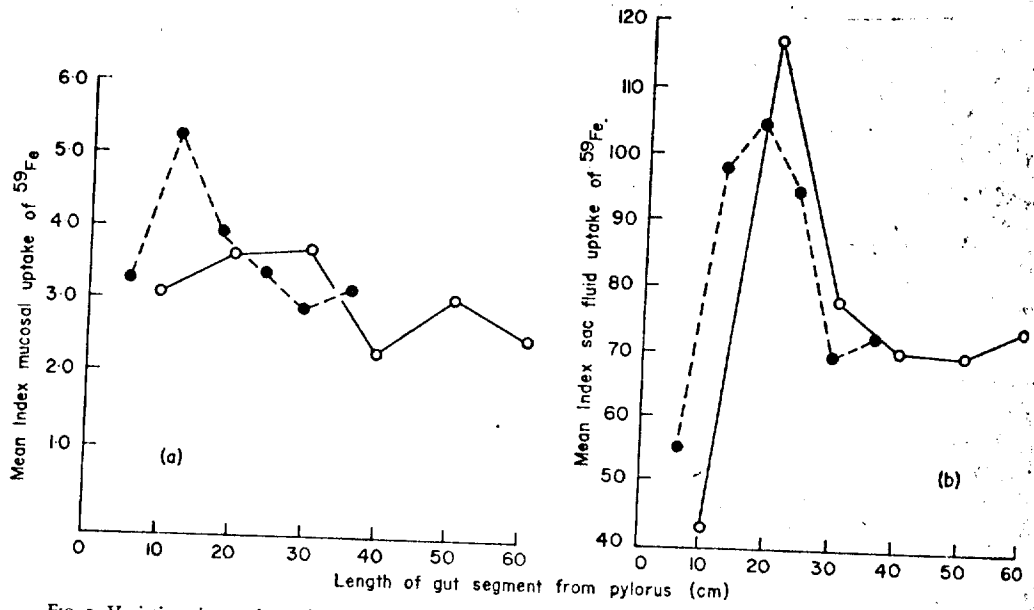

FIG. 1. Variations in uptake and transport of ^{59}Fe along the rat small intestine. The values shown are the means derived from nine rats studied using 6 cm. sacs (●) and six rat studies with 10 cm. sacs (○). (a) Mucosal uptake; (b) sac fluid uptake.

haemochromatosis group, but the means were not significantly different ($P > 0.05$). Histological preparations showed an intact mucosa without cellular degeneration at the end of the experiment. No correlation could be found between the values of iron absorption and the iron uptake of rat gut sacs exposed to the gastric juice of seven patients with haemochromatosis (Table III).

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Site and Nature of Mucosal Uptake

In other experiments either sequential 6 cm. sacs (in nine rats) or 10 cm. sacs (in six rats) were formed and 0.2 ml. 10 per cent ascorbic acid and ^{59}Fe ferrous ascorbate were used in place of ^{59}Fe ferric citrate. The effect of the increased amount of ascorbic acid was to greatly augment uptake of ^{59}Fe by the sac fluid. Peak uptake occurred in the second 10 cm. sac and the third 6 cm. sac, corresponding to the upper jejunum (Fig. 1). The lowest rates were seen in the duodenum. Mucosal uptake rates showed no striking difference to those obtained using ^{59}Fe ferric citrate. 6 cm. sacs were also incubated for 1, 2 or 3 hours in mannose solution, containing ^{59}Fe ferrous ascorbate but no added ascorbic acid. There was a notable

TABLE IV

EFFECT OF VARYING INCUBATION TIMES ON UPTAKE OF RADIO-IRON BY EVERTED GUT SACS

No. of tests	*Hours of incubation*	*Mean index sac fluid uptake of ^{59}Fe*	*Mean index mucosal uptake of ^{59}Fe*
2	1	36.2	4.87
1	2	304.2	19.97
3	3	254.7	66.88

increase in transport of iron to the inside of the gut sac when incubation was continued for 2 hours, but little change if incubation was continued for a further hour, suggesting that death of the sac had occurred by this time. The greater the duration of incubation, the greater the amount of iron taken up by the mucosa, as would happen in a passive transfer system (Table IV).

DISCUSSION

The results reported here do not confirm Murray's 1968 findings of a factor in neutralized gastric juice from patients with haemochromatosis or iron deficiency which is capable of potentiating iron absorption by rat gut sacs or segments. Mignon, Russell, Semb, Morgan, Finch and Nyhus (1965) have also been unable to repeat Koepke's findings in the dog. In man, both a Cardiff group (Jacobs, Rhodes and Eakins, 1967) and South African workers (Jacobs, Charlton and Bothwell, 1968) were unable to show a stimulatory effect of gastric juice obtained from patients with iron deficiency on iron absorption. Although Turnberg (1968) has reported studies in a few patients showing that concentrated neutralized gastric juice from patients with iron deficiency anaemia was capable of increasing iron absorption in the normal subject, he measured iron absorption by the stool collection technique, which can be unreliable. Recently, Jacobs (1968) showed that neutralized gastric juice would potentiate absorption of radio-iron in patients with complete gastric atrophy, possibly by facilitating the digestion and release of iron in an absorbable form, but he concluded that this was unlikely to be a physiological mechanism in the control of iron absorption.

Other studies in this laboratory have failed to confirm the presence of a specific iron binding protein in gastric juice (Wynter and Williams, 1968). Iron was bound loosely by a

gastric juice component, possibly a glycoprotein, to a similar extent in control subjects and in patients with iron deficiency anaemia or haemochromatosis. There is indirect evidence, mainly obtained from studies in which pancreatin has been fed, suggesting that there is a factor in pancreatic juice normally inhibiting iron absorption (Biggs and Davis, 1963). The most recent and detailed investigation of the effects of pancreatin in iron absorption, however, would suggest that this is not so (Kavin, Charlton, Jacobs, Green, Torrance and Bothwell, 1967), and current evidence would point to mucosal, rather than luminal, abnormalities in idiopathic haemochromatosis.

Schachter and his co-workers (Dowdle, Schachter and Schenker, 1960), using the everted sac technique, have shown that iron transfer will occur against a concentration gradient, is dependent on energy provided by phosphate bonds and can be inhibited by metabolic poisons. They also showed that transfer took place by a two-step mechanism with a rapid uptake by the mucosa followed by slower transfer to the serosal surface (Manis and Schachter, 1962). However, they incubated gut sacs for 3 hours, and the present studies would suggest that the mucosa may not retain cellular function for this time. We could find no evidence for active transport of iron *in vitro*. Brown and Justus (1958) and Pearson and Reich (1965) also showed that iron diffused across the gut wall by a passive process *in vitro*, which was not affected by substitution of nitrogen for oxygen. Both Dowdle *et al.* (1960) and Pearson and Reich (1965) found that ascorbic acid increased iron uptake and this study has confirmed their findings, sac fluid uptake being more strikingly enhanced than mucosal uptake. Ascorbic acid may act by virtue of its ability to reduce ferric to ferrous ions, or partly by reducing the pH of intestinal contents, for a similar increase in transport has been achieved with hydrochloric acid and nitric acid *in vivo* (Jacobs *et al.*, 1966).

This paper has also failed to confirm Schachter's descriptions of a markedly enhanced iron uptake by the duodenum *in vitro*, with a gradual decline down the jejunum and ileum. Pearson and Reich (1965) found that uptake of iron was least in the first of their 6 cm. sacs, and that in all other gut sacs uptake was uniform. However, Brown and Justus (1958), who described equal uptake in all segments of the rat's small intestine *in vitro*, detected a distinct gradient, maximal in the duodenum, when iron was given orally *in vivo*. The latter may be explained by studies on rats by Jacobs *et al.* (1966), which related enhancement of duodenal iron absorption to a lowering of luminal pH, the duodenum being exposed to gastric hydrochloric acid in the intact animal. Iron absorption in the intact subject undoubtedly involves a number of interrelated processes, and it is this that makes their experimental study so difficult.

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MODIFICATION OF ASCORBIC ACID IN THE BLOOD, IN THE ADRENALS, AND IN THE LIVER UNDER THE INFLUENCE OF THE ADMINISTRATION OF ALLOXAN IN RATS

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SUMMARY

Research has been carried out, at different intervals of time (1-72 hrs.), on ponderal variations of the adrenals and modifications of glycemia and the ascorbic acid of the blood, the adrenals and the liver caused by the administration of alloxan in doses of 100 and 200 mg/kg of body weight to white rats. Under the influence of a large dose, the vitamin C of the blood, after a slight decrease during the first hour, is increased greatly (12-72 hrs.). A small dose causes a less marked increase.

Under the influence of a large dose of alloxan, the ascorbic acid content of the adrenal reaches the maximum after twelve hours, after which it tends to return slowly to normal values. In the liver the increase is observed from the very first hour with a return to normal at 72 hours. A small dose of alloxan causes an obvious increase of the values of ascorbic acid in the adrenals for the whole experimental period.

In the adrenal there appear aspects of fascicularization and a decrease of the lipids at the level cortical accompanied by a ponderal increase of the gland. There is a discussion of the diencephalo-hypophyso-cortico-adrenal stress-inducing effect of alloxan supported also by the fact that the adrenal modifications were proportional to the dose. (END OF SUMMARY)

Increased attention has been given in recent years to the interrelationships between the stress states and the various forms of diabetes, including that which is caused experimentally by the administration of alloxan (1), (9), (12), (13), (14), (17). In this regard the fact has been noted that, along with degenerative lesions of the pancreas, alloxan causes, in the first 72 hours after administration, a series as well of other modification, interpreted as a stress-inducing effect of the substance (8), (11), (23).

There are, however, authors who consider that the stress-inducing effect of alloxan is not due to its own effect properly speaking, but to a decrease of ascorbinemia, since the administration of vitamin C can prevent it (3), (5), (6). The histo-pathological and histo-chemical aspects of the adrenals are characteristic modifications encountered in the course of stress (3).

Contradictory data also exist . Thus the ascorbic acid and cholesterol content of the adrenal has been found to be increased, decreased (8), or even unchanged (13).

Because, in the literature consulted, we have not found data pertaining to modification of ascorbinemia parallel with those of the vitamin C content of the adrenal and the liver, it appears interesting to us to study this relationship dynamically, for 1-72 hrs. respectively.

In order to evaluate to what degree it is a question of the stress-inducing effect of the toxic element itself, we have used alloxan in two different doses (which within a longer or shorter time the appearance of diabetes.)

MATERIAL AND METHOD

Our studies were carried out on 82 adult white rats of both sexes and were carried out between 1 and 72 hours after administration of alloxan.

The animals were divided into three groups.

Group A included a single lot of 24 animals and served as a control (lot I).

Group B included 21 animals divided into three lots (II,III, IV) killed after 12, 24, and 72 hours, respectively after administration of alloxan in a dose of 100 mg/kg of body weight.

Group C, consisting of 6 lots (V-X), included 34 animals killed at 1,3,6,12,24, and 72 hours, respectively after administration of alloxan in a dose of 200 mg/kg of body weight.

There was used a five percent alloxan solution (Prolabo, Roche), prepared extemporaneously, in distilled water. The injection was made intramuscularly into animals deprived of food for 24 hours. The blood was collected after the killing of the rats by decapitation.

A study was made of glycemia and ascorbinemia, and the vitamin C in the liver and the left adrenal was determined while the right adrenal served for histo-pathological studies.

We evaluated the ponderal modifications of the adrenals by weighing regularly the left adrenal gland and by determining the ratio between the weight of the adrenal/the body weight/100, which we have named the ponderal index of the adrenal.

The measurement of vitamin C was carried out in the blood and in the tissues according to the Roe-Kuther (21) method, but the measurement of glycemia was effected by the Hagedorn-Jensen technique. The values obtained were reported in mg per 100 ml

for the blood and per 100 g of fresh tissue for the adrenal and the liver.

After removal, the adrenals were fixed in 10% salted formalin, and afterward were enclosed in paraffin and dissected to 4 μ for the general aspect, the dyeing was carried out with hemalin -eosin.

The detection of ascorbic acid was done by fixation in 5% silver azotate according to the Bacchus method (cited after [19]).

RESULTS

The data obtained vary in relationship to the dose of alloxan injected (tables 1 and 2).

Modifications of glycemia. One hour after administration of alloxan (200 mg) glycemia greatly increased (1.26 vis-à-vis 0.73 g%), While at three hours it decreases to 0.53 g %. Finally, glycemia increased progressively; after 6 hours to 0.71 g %; after 12 hours to 1.44 g %; after 24 hours to 2.09 g %, at 72 hours reaching very high values, i.e. 10.10 g %.

Glycemia underwent smaller modifications in the case of the injection of alloxan in a small dose (100 mg/kg of body weight). Thus, after 12 or 24 hours, the values are 0.66 and 0.90 g % respectively. At 72 hours, an obvious hyper-glycemia (1.26 g %) is produced, but very much lower than that found after administration of the large dose.

TABLE I: Ponderal variations of the adrenals, variations of glycemia and ascorbic acid in the blood, adrenals and liver at different intervals of time after the administration of alloxan in a dose of 100 mg/kg of body weight (mean values).

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Tabelul nr. 1

Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sânge, suprarenale și ficat la diferite intervale de timp de la administrarea de aloxanului în doză de 100 mg/kg corp (valori medii)

Grupa și lotul de animale	Nr. animale	Greutatea animalelor g	Tratamentul	Timpul ore	Glicemia g‰	Greutate SR mg	Greutate SR mg% (IP)	Acid ascorbic în sânge mg%	Acid ascorbic în SR mg% (țesut proaspăt)	Acid ascorbic în ficat mg% (țesut proaspăt)
A I	24	173	–	–	0,73	12,8	7,36	0,4	344	–
B II	9	179	aloxan	12	0,66	9,66	5,51	2,52	519	–
III	4	150	aloxan	24	0,90	12,10	8,09	–	510	–
IV	11	181	aloxan	72	1,26	14,41	7,49	2,20	549	–

TABLE II: Ponderal variations of the adrenals, variations of glycemia and ascorbic acid in the blood, adrenals and liver at various intervals of time after the administration of alloxan in a dose of 200 mg/kg of body weight (mean values).

Tabelul nr. 2

Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sânge, suprarenale și ficat la diferite intervale de timp de la administrarea aloxanului în doză de 200 mg/kg corp (valori medii)

Grupa și lotul de animale	Nr. animale	Greutatea animalelor g	Tratamentul	Timpul ore	Glicemia g‰	Greutate SR mg	Greutate SR mg% (IP)	Acid ascorbic în sânge mg%	Acid ascorbic în SR mg% (țesut proaspăt)	Acid ascorbic în ficat mg% (țesut proaspăt)
A I	24	173	–	–	0,73	12,8	7,36	0,40	344	23,9
C V	7	144	aloxan	1	1,26	13,4	9,30	0,29	351	35,2
VI	4	135	aloxan	3	0,53	16,1	11,93	–	344	–
VII	5	137	aloxan	6	0,71	10,16	7,42	–	422	–
VIII	7	162	aloxan	12	1,41	10,20	6,29	2,8	577	37,7
IX	7	145	aloxan	24	2,09	10,70	7,38	10,8	481	34,3
X	4	163	aloxan	72	10,10	21,30	13,00	10,8	365	21,5

Ponderal modification of the adrenals. As is observed in tables 1 and 2 as well as in figures 1 and 2, the injection of alloxan caused ponderal variations of the adrenals reflected as well in the relationship studied by us. In the case of the large dose of alloxan, after one hour and after three hours, the weight of the adrenals is more increased in comparison with the values encountered at normal (9.30 and 11.93 respectively compared with 7.36 mg %).

12 hours after the injection of alloxan and regardless of the dose injected, the ratio shows more decreased values in comparison with the normal (5.51 and 6.29 mg % respectively).

After 24 hours, the ponderal index indicates a return to the values of the control-lot (7.38 mg %) and reaches the greatest value, 72 hours after administration of the toxic element in a large dose (13.00 mg %).

At the same time, the animals injected with a small dose of alloxan show a more noteworthy increase at 24 hours, while after 72 hours a tendency toward a decrease is shown, approaching normal values (8.09 and 7.49 mg % respectively).

Variations in ascorbic acid. In the blood, with the exception of the first hour (table 2), ascorbinemia shows noteworthy increases regardless of the dose of alloxan administered (tables 1 and 2). We note however the fact that in the case of the administration of a large dose, both at 24 and at 72 hours, the values for ascorbic acid found in the blood reach extremely large amounts more than 10 mg % (table 2).

At the level of the adrenals, vitamin C undergoes less marked variations in the case of injection of alloxan in a large dose. A more significant increase can be observed of course twelve hours after administration of the toxic element when the quantity encountered is more than 65 % greater than that observed in the animal-controls (table 2).

With regard to the small dose of alloxan, this caused constant increases in the amounts of ascorbic acid in the adrenals, which varied between 47 and 60% compared with the value encountered in the animals of the control-lot (table 1).

At the level of the liver (determination carried out only in the case of a large dose), vitamin C was found to be constantly increased at one hour, 12, and 24 hours and returned to normal values 72 hours after the administration of the alloxan (table 2).

The histo-pathological and histo-chemical modifications [1] showed us that, under the influence of alloxan, alterations are produced of the cyto-architecture of the cortico-adrenal, which especially affect the fasciculated and reticulated zones.

Histologically, the cortico-adrenal presents an increased total surface, with obliteration of the interlaminar limit between the reticulated and the fasciculated, the latter being the seat of a hypertrophico-hyperplasic process of variable intensities. The glomerular is partially consumed in the process of fascicularization.

With regard to ascorbic acid, the histo-chemical studies do not allow us certain accurate quantitative evaluations. Nevertheless,

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[1] Şneer, Brătianu, Stroia, Herşcovici, Morpho-pathological cortico-adrenal modifications under the Influence of the administration of alloxan (on the press).

the fact is noted that while vitamin C is found in the control animals, as also in research by Petrea (20), equally distributed in all the cortico-adrenal zones, under the influence of the alloxan, the ascorbic acid appe .s in the form of uneven (unequal) granulations, distributed unsystematically in the whole cortical, especially in the fasciculated and reticulated.

DISCUSSION OF THE RESULTS

The results obtained by us confirm the principal data of the literature with regard to modifications produced by the injection of alloxan. In this sense, we can cite especially the variations in glycemia (15), as well as modifications of the adrenals. both from the ponderal point of view and from the histopathological aspects (8), (11), (23).

The study of the effect of alloxan under the influence of two doses confirmed the assumption that the toxic element will exert a more stress-inducing effect as the quantity injected is greater. In this aspect, the results obtained show a ponderal increase of the adrenals that is more evident in the case of the injection of alloxan in a dose of 200 mg/kg of body weight (table 2; figure 1).

In agreement with the data from the literature and especially with the series of studies by Milcu and Simionescu (16) regarding the compensatory hypertrophy of the cortico-adrenal, we have interpreted the ponderal increase of the adrenals as a consequence of their stimulation through the discharge of CRF (22) and of the endogenous cortico-trophin, respectively. In this sense, it can be observed that, after a brief hyperfunction in the first three

hours, due probably to the stress-inducing effect of alloxan (2), (8), a decrease in activity begins which is minimal for 12 hours, followed again by an increase in it reflected in an increase of weight of the adrenals. The curve described fits, we believe, into the general response of the adrenals to aggression.

From our results, we observed that ascorbinemia, with the exception of a decrease of 28% compared with the normal value, which we found as did Siliprandi (24) as well, in the first hour after the injection of alloxan, not only not to have decreased but, on the contrary, to have increased to values of more than 2600% .

We are not acquainted with data with regard to vitamin C in the liver. Under the conditions of our experiments, ascorbic acid increased between one hour and 24 hours, returning to normal values 72 hours after injection of alloxan (fig. 3).

The fact could be interpreted as an adaptation reaction to the effect of the toxic element, either through the temporary depositing of an increased amount of vitamin C in the liver or as a consequence of a greater local synthesis of ascorbic acid or even through both mechanisms.

With regard to the ascorbic acid content of the adrenal, the data from the literature indicate every possible variation, increases, decreases, or a lack of any modification whatever caused by alloxan (13).

It might be possible that the divergences indicate the fact that the evaluation was made especially by histo-chemical methods, but, as we have mentioned, this did not allow us to distinguish quantitative modifications which the biochemical measurements

indicated.

The biochemical determinations indicated to us that the ascorbic acid content of the adrenals undergoes variations over a period of time, but a quantity smaller than that of the control-animals was never encountered. On the contrary, we have even been able to observe an increase of 60 and 40 percent respectively in these values at 12 and 24 hours after the injection of alloxan in a large dose, while at 72 hours, we witness a return to normal values. In the case of a small dose, the increase in quantity of vitamin C of the level of the adrenals is maintained constant between 12 and 72 hours inclusively.

Our data therefore show that, in the production of stress in animals injected with alloxan, in no single case could a marked decrease of ascorbic acid in the blood be held to blame since, with the exception of a minimum value at the first hour, the ascorbinemia was clearly increased in every case.

Nevertheless, it has not been explained how the injection of vitamin C 24 hours after the administration of alloxan prevents, as indicated by Chatterjee (3), the hypertrophy of the adrenal encountered by us as well after 72 hours. On the other hand, in another series of studies, we have observed that the injection of vitamin C in a large dose appears to stimulate the cortico-adrenal activity (27).

The fact noted by Devecerski and Frawley (8) that, in hypophysectomized rats, alloxan does not cause hyper-activity of the cortico-adrenal, suggests that the substance does not act directly on the adrenal.

If to this, we add as well the observations by Saba and Hoet (11), (23), who, blocking the hypophysis with fluorocortisol, reduce the pancreatic lesions and impede the appearance of hyper-glycemia as well as the increase of the steroidogenesis produced by alloxan, we can admit that, through definite disturbances by this toxic element, the exerting of action by the substance can also be observed, an action through the intermediary of the hypophyso-cortico-adrenal axis.

The various ponderal modifications of the adrenals observed in those two groups of rats injected with a small or large dose of alloxan cause us to believe that they form a further argument in support of what was stated above.

The fact that, under the conditions of the discharge of ACTH, the vitamin C at the level of the adrenals does not decrease, as we might have expected, could be explained by the fact that the blood which irrigates the gland has an increased ascorbic acid content (as indicated by our experiments) or by some modification in transportation(circulation) (18). In this way, we have been able to understand as well the differences between our results, in which alloxan did not cause the depletion of ascorbic acid, and the studies made by P. Dere nco and collaborators (7), who largely studied the hypophyso-cortico-adrenal activity under numerous conditions of drug-induced (pharmacological) stress.

Finally, it would be more necessary that we find an explanation of the causes which determine an increase in the amounts of vitamin C at the level of the blood.

It would be possible that the alloxan itself or some metabolites that resulted from its action upon the tissues behaves as reducing substances similar to ascorbic acid. Both the fact however that one hour after the injection of alloxan, the amount of ascorbic acid in the adrenal is not modified and the ascorbic acid in the blood even is decreased, and the technique of measuring used by us make the supposition mentioned above not very probable.

It would be possible that alloxan- and this if it is proved, does not appear more interesting to us - stimulates the synthesis of vitamin C. As our research was carried out on rats which we know can synthesize ascorbic acid, it would make this hypothesis more probable.

In this sense we could understand as well the modifications observed in the first hour after the administration of alloxan. Thus, while vitamin C in the blood decreases and vitamin C in the adrenal is not modified, the ascorbic acid content of the liver greatly increases (fig. 3). Thus, as we have further indicated (27), the synthesis and the depositing of vitamin C would be effected especially at the level of the liver.

Numerous problems still inadequately clarified, contradictions that still persist, as well as the importance which the adrenal has in general in the metabolic activity of the organism and certainly also in the disturbances of the picture of diabetes, justify and suggest the need for new research.

CONCLUSIONS

Alloxan administered in doses of 100 and 200 mg/kg of body weight caused various modifications. Under the influence of a

large dose, the vitamin C in the blood, after it shows a slight decrease in the first hour, greatly increases (12-72 hrs.). A small dose causes a ~~much~~ less marked increase.

Under the influence of a large dose, the ascorbic acid content of the adrenal reaches the maximum at 12 hours, after which it has a tendency to return slowly to normal value. A small dose produces an obvious increase, which is maintained during the whole experimental period.

The ascorbic acid content of the liver even increases from the first hour and returns to normal after 72 hours (large dose).

The adrenal shows modifications which give evidence of the discharge of endogenous ACTH, characterized by a ponderal increase, aspects of fascicularization, and a decrease of the lipids of the level of the cortical.

Figure 1: Ponderal variations of the adrenals, variations of glycemia and of ascorbic acid in the blood and adrenals after the administration of alloxan (200 mg/kg of body weight; mean values).

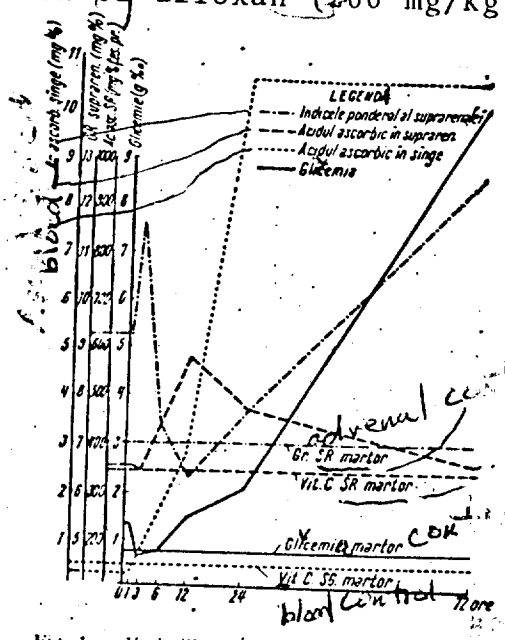


Fig. 1. — Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sînge și suprarenale după administrarea aloxanului (200 mg/kgcorp; valori medii).

Figure 2: Ponderal variations of the adrenals, variations of glycemia and of ascorbic acid in the blood and adrenals after the administration of alloxan (100 mg/kg of body weight; mean values).

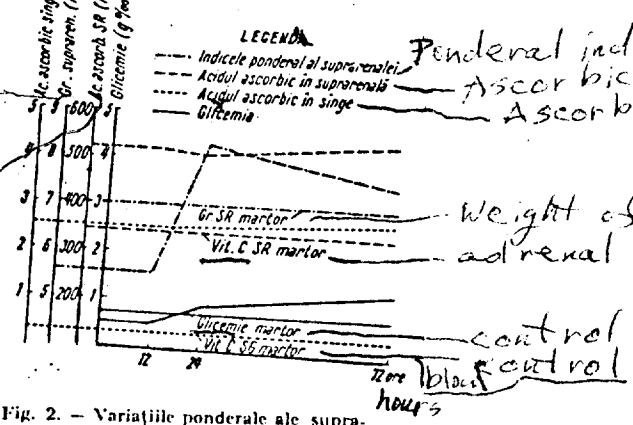
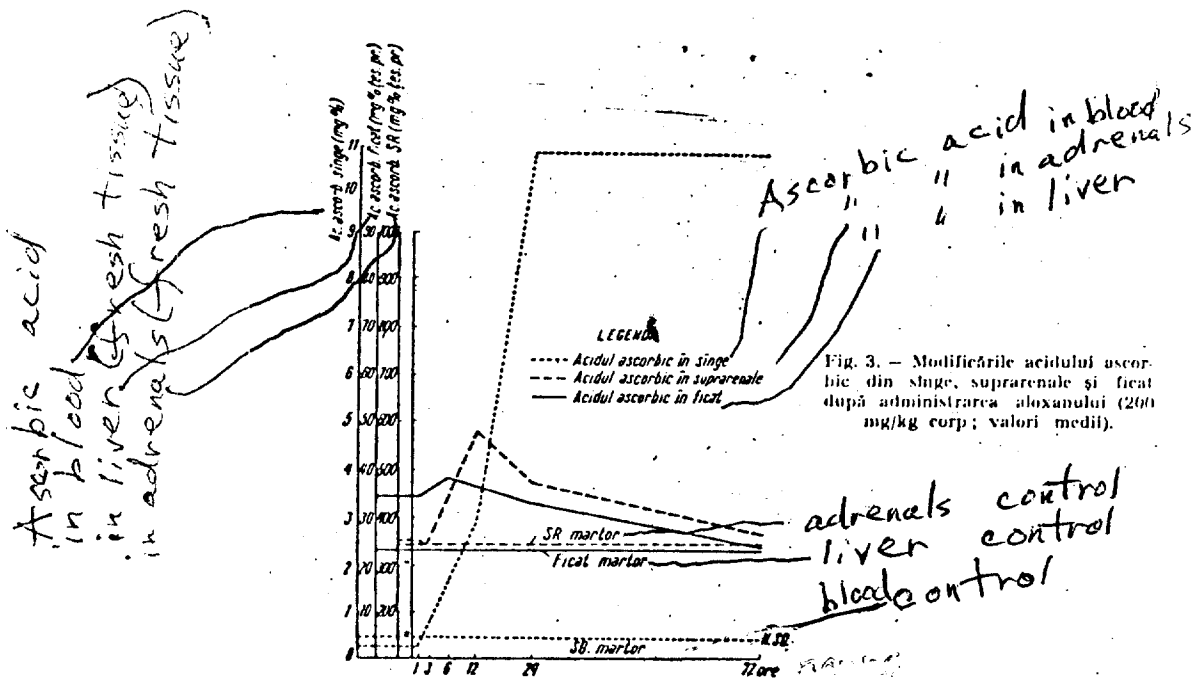


Fig. 2. — Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sînge și suprarenale după administrarea aloxanului (100 mg/kg corp; valori medii).

Figure 3: Modifications of ascorbic acid in the blood, adrenals, and the liver after the administration of alloxan (200 mg/kg of body weight; mean values).



MODIFICĂRILE ACIDULUI ASCORBIC ÎN SÎNGE, SUPRARENALE ŞI FICAT SUB INFLUENŢA ADMINISTRĂRII ALOXANULUI LA ŞOBOLANI*

A. ŞNEER, VERONICA STROIA, B. HERŞCOVICI, ELENA CONSTANTIN şi EMILIA PAPP

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S-au cercetat, la diferite intervale de timp (1—72 de ore), variaţiile ponderale ale suprarenalei şi modificările glicemiei şi ale acidului ascorbic din sînge, suprarenale şi ficat determinate de administrarea aloxanului în doze de 100 şi 200 mg/kg corp la şobolanii albi. Sub influenţa dozei mari, vitamina C din sînge, după o uşoară scădere în prima oră, creşte foarte mult (12—72 de ore). Doza mică determină o creştere mai puţin marcată.

Sub influenţa dozei mari de aloxan conţinutul în acid ascorbic al suprarenalei atinge maximul la 12 ore, după care are tendinţa de a reveni lent la valorile normale. În ficat, creşterea se constată chiar din prima oră, cu o revenire la normal la 72 de ore. Doza mică de aloxan determină creşterea evidentă a valorii acidului ascorbic în suprarenale pentru toată perioada experimentală.

În suprarenală apar aspecte de fascicularizare şi scădere a lipidelor la nivelul corticalei însoţite de creşterea ponderală a glandei. Se discută acţiunea stressantă diencefalo-hipofizo-corticosuprarenală a aloxanului, susţinută şi de faptul că modificările suprarenale au fost proporţionale cu doza.

O atenţie crescută a fost acordată în ultimii ani interrelaţiilor dintre stările de stress şi diversele forme de diabet, inclusiv cel experimental determinat de administrarea aloxanului (1), (9), (12), (13), (14), (17). În această privinţă s-a remarcat faptul că, pe lîngă leziunile degenerative pancreatice, aloxanul determină, în primele 72 de ore de la administrare, şi o serie de alte modificări, interpretate ca o acţiune stressantă a substanţei (8),(11),(23).

Sînt însă autori care consideră că efectul stressant al aloxanului nu ar fi datorat acţiunii sale propriu-zise, ci unei scăderi a ascorbinemiei, administrarea vitaminei C putîndu-l preveni (3), (5), (6). Aspectele histopatologice şi histochimice ale suprarenalelor sînt caracteristice modificărilor întîlnite în cursul stressului (3).

* Primit în redacţie la data de 13 iunie 1969.

Există şi date contradictorii. Astfel conţinutul suprarenalei în acid ascorbic şi colesterol a fost găsit crescut, scăzut (8) sau chiar nemodificat (13).

Deoarece în literatura consultată nu am găsit date care să fi urmărit modificările ascorbinemiei paralel cu cele ale conţinutului în vitamina C al suprarenalei şi al ficatului ni s-a părut interesant a cerceta această relaţie, dinamic, respectiv, între 1 şi 72 de ore.

Pentru a aprecia în ce măsură este vorba de acţiunea stressantă a toxicului însuşi, am utilizat aloxanul în două doze diferite (care determină într-un timp mai lung sau mai scurt apariţia diabetului).

MATERIAL ŞI METODĂ

Cercetările noastre au fost efectuate pe un număr de 82 de şobolani albi adulţi de ambele sexe şi s-au desfăşurat între 1 şi 72 de ore de la administrarea aloxanului.

Animalele au fost împărţite în trei grupe.

Grupa A a cuprins un singur lot de 24 de animale şi a servit pentru control (lot I).

Grupa B a cuprins 21 de animale împărţite în trei loturi (II, III şi IV) sacrificate la 12, 24 şi, respectiv, 72 de ore după administrarea aloxanului în doză de 100 mg/kg corp.

Grupa C, formată din 6 loturi (V—X), a cuprins 34 de animale sacrificate la 1, 3, 6, 12, 24 şi, respectiv, 72 de ore, după administrarea aloxanului în doză de 200 mg/kg corp.

S-a folosit o soluţie de 5% aloxan (Prolabo Roche), preparat extemporaneu, în apă distilată. Injectarea s-a făcut intramuscular, la animale aflate la post timp de 24 de ore. Sîngele s-a recoltat după sacrificarea şobolanilor prin decapitare.

S-au urmărit glicemia şi ascorbinemia şi s-a determinat vitamina C în ficat şi în suprarenala stîngă, în timp ce glanda din dreapta a servit pentru cercetări histopatologice.

Modificările ponderale ale suprarenalelor le-am apreciat prin cîntărirea cu regularitate a glandei din partea stîngă şi prin determinarea raportului dintre greutatea suprarenalei/greutatea corpului × 100, pe care l-am denumit indice ponderal al suprarenalei (IPSR).

Dozarea vitaminei C s-a efectuat în sînge şi în ţesuturi după metoda Roe-Kuther (21), iar a glicemiei prin tehnica Hagedorn-Jensen. Valorile obţinute au fost raportate în mg la 100 ml pentru sînge şi la 100 g de ţesut proaspăt pentru suprarenale şi ficat.

După prelevare, suprarenalele au fost fixate în formol sărat 10%, iar apoi au fost incluse în parafină şi secţionate la 4 μ pentru aspectul general, s-a efectuat coloraţia cu hemalaun-eozină.

Evidenţierea acidului ascorbic s-a făcut prin fixare în azotat de argint 5% după metoda Bacchus (citat după (19)).

REZULTATE

Datele obţinute sînt diferite în funcţie de doza de aloxan injectată (tabelele nr. 1 şi 2).

Modificările glicemiei. După o oră de la administrarea aloxanului (200 mg) glicemia a crescut mult (1,26 faţă de 0,73 g‰), pentru ca la 3 ore să scadă pînă la 0,53 g‰. Ulterior, glicemia a crescut progresiv; după 6 ore la 0,71 g‰; după 12 ore la 1,44 g‰; după 24 de ore la 2,09 g‰, la 72 de ore atingînd valori extrem de mari, şi anume 10,10 g‰.

Glicemia a suferit modificări mai reduse în cazul injectării aloxanului în doză mică (100 mg/kg corp). Astfel, după 12 sau 24 de ore, valorile

Stud. Cercet. Endocrinol. 21(2): 137-145, 1970

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Tabelul nr. 1

Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sînge, suprarenale și ficat la diferite intervale de timp de la administrarea de aloxanului în doză de 100 mg/kg corp (valori medii)

Grupa și lotul de animale	Nr. animale	Greutatea animalelor g	Tratamentul	Timpul ore	Glicemia g‰	Greutate SR		Acidul ascorbic în:		
						mg	mg% (IP)	sînge mg%	SR mg% țesut proaspăt	ficat mg% țesut proaspăt
A I	24	173	—	—	0,73	12,8	7,36	0,4	344	—
B II	9	179	aloxan	12	0,66	9,66	5,51	2,52	519	—
III	4	150	aloxan	24	0,90	12,10	8,09	—	510	—
IV	11	181	aloxan	72	1,26	14,41	7,49	2,20	549	—

Tabelul nr. 2

Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sînge, suprarenale și ficat la diferite intervale de timp de la administrarea aloxanului în doză de 200 mg/kg corp (valori medii)

Grupa și lotul de animale	Nr. animale	Greutatea animalelor g	Tratamentul	Timpul ore	Glicemia g‰	Greutate SR		Acidul ascorbic în:		
						mg	mg% (IP)	sînge mg%	SR mg% țesut proaspăt	ficat mg% țesut proaspăt
A I	24	173	—	—	0.73	12,8	7,36	0.40	344	23,9
C V	7	144	aloxan	1	1,26	13,4	9,30	0,29	351	35,2
VI	4	135	aloxan	3	0,53	16,1	11,93	—	344	—
VII	5	137	aloxan	6	0.71	10,16	7,42	—	422	—
VIII	7	162	aloxan	12	1,44	10,20	6,29	2,8	577	37,7
IX	7	145	aloxan	24	2,09	10,70	7,38	10,8	484	34,3
X	4	163	aloxan	72	10,10	21,30	13,00	10,8	365	24,5

sînt de 0,66 și, respectiv, de 0,90 g‰. La 72 de ore se produce o hiperglicemie evidentă (1,26 g‰), dar mult inferioară celei găsite după administrarea dozei mari.

Modificările ponderale ale suprarenalelor. Așa cum se observă din tabelele nr. 1 și 2, precum și din figurile 1 și 2, injectarea aloxanului a provocat oscilații ponderale ale suprarenalelor reflectate și în raportul urmărit de noi (IPSR). În cazul dozei mari de aloxan, după o oră și după 3 ore, greutatea suprarenalelor este mai crescută în comparație cu valorile găsite la normali (9,30 și, respectiv, 11,93 față de 7,36 mg%).

După 12 ore de la injectarea aloxanului și indiferent de doza injectată, raportul arată valorile cele mai reduse în comparație cu normalul (5,51 și, respectiv, 6,29 mg%).

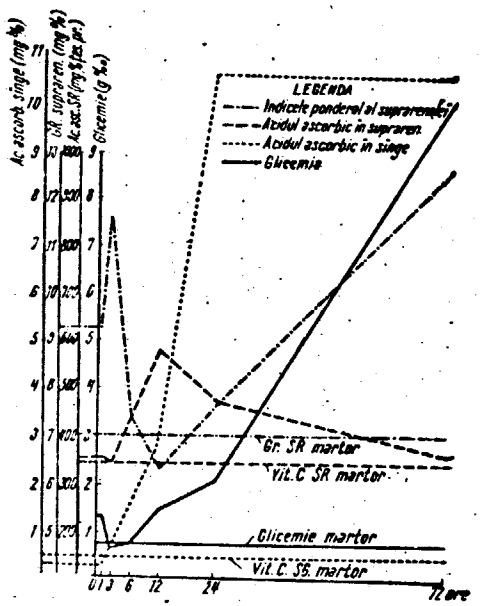

Fig. 1. — Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sînge și suprarenale după administrarea aloxanului (200 mg/kgcorp; valori medii).

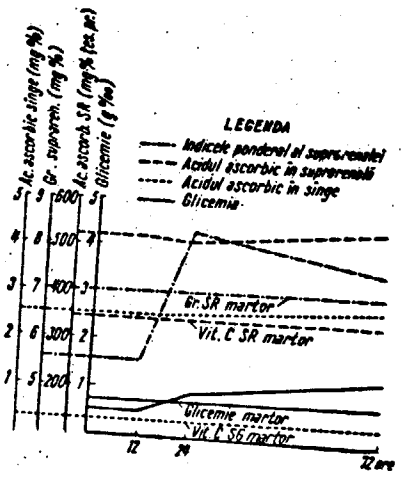

Fig. 2. — Variațiile ponderale ale suprarenalelor, ale glicemiei și ale acidului ascorbic din sînge și suprarenale după administrarea aloxanului (100 mg/kg corp; valori medii).

După 24 de ore, indicele ponderal marchează o revenire la valorile lotului-martor (7,38 mg%) și atinge valoarea cea mai mare după 72 de ore de la administrarea toxicului în doză mare (13,00 mg%).

În același timp, animalele injectate cu doză mică de aloxan prezintă o creștere mai însemnată la 24 de ore, pentru ca după 72 de ore să manifeste o tendință de scădere, apropiindu-se de valorile normale (8,09 și, respectiv, 7,49 mg%).

Variațiile acidului ascorbic. În sînge, cu excepția primei ore (tabelul nr. 2), ascorbinemia marchează creșteri însemnate indiferent de doza de aloxan administrată (tabelele nr. 1 și 2). Remarcăm însă faptul că în cazul administrării dozei mari, atît la 24, cît și la 72 de ore, valorile de acid ascorbic găsite în sînge ating cantități extrem de mari, peste 10 mg% (tabelul nr. 2).

La nivelul suprarenalelor, vitamina C suferă oscilații puțin marcate în cazul injectării aloxanului în doză mare. O creștere mai însemnată se poate observa doar după 12 ore de la administrarea toxicului, cînd

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cantitatea găsită este cu peste 65% mai mare decît cea constatată la animalele-martor (tabelul nr. 2).

În ceea ce privește doza mică de aloxan, aceasta a determinat creșteri constante ale cantității de acid ascorbic din suprarenale, care au variat între 47 și 60% față de valoarea găsită la animalele din lotul martor (tabelul nr. 1).

La nivelul ficatului (determinări efectuate numai în cazul dozei mari), vitamina C a fost găsită constant crescută la o oră, 12 și 24 de ore și a revenit la valorile normale după 72 de ore de la administrarea aloxanului (tabelul nr. 2).

Modificările histopatologice și histochimice[1] ne-au arătat că sub influența aloxanului se produc alterări ale citoarhitectonicii corticosuprarenalei, care afectează în special zonele fasciculată și reticulată.

Histologic, corticosuprarenala prezintă o suprafață totală sporită, cu ștergerea limitei interlaminare dintre reticulată și fasciculată, ultima fiind sediul unui proces hipertrofico-hiperplazic de intensități variabile. Glomerulara este parțial consumată în procesul de fascicularizare.

În privința acidului ascorbic, cercetările histochimice nu ne permit aprecieri certe cantitative. Se remarcă totuși faptul că în timp ce vitamina C se găsește la animalele-martor, ca și în cercetările lui Petrea (20), egal repartizată în toate zonele corticosuprarenalei, sub influența aloxanului acidul ascorbic apare sub formă de granulații inegale, distribuite nesistematic în toată corticala, cu deosebire în fasciculată și reticulată.

DISCUȚIA REZULTATELOR

Rezultatele obținute de noi confirmă principalele date din literatură privind modificările produse de injectarea aloxanului. În acest sens putem cita îndeosebi variațiile glicemiei (15), precum și modificările suprarenalelor atît din punct de vedere ponderal, cît și al aspectelor histopatologice (8), (11), (23).

Cercetarea acțiunii aloxanului sub influența a două doze a confirmat presupunerea că toxicul va exercita un efect cu atît mai stressant, cu cît cantitatea injectată va fi mai mare. Sub acest aspect rezultatele obținute arată o creștere ponderală a suprarenalelor mai evidentă în cazul injectării aloxanului în doză de 200 mg/kg corp (tabelul nr. 2; fig. 1).

În acord cu datele din literatură și îndeosebi cu suita de cercetări ale lui Milcu și Simionescu (16) privind hipertrofia de compensație a corticosuprarenalei am interpretat creșterea ponderală a suprarenalelor ca o consecință a stimulării lor prin descărcarea CRF (22) și, respectiv, a corticotropului endogen. În acest sens, se poate constata că, după o scurtă hiperfuncție din primele 3 ore, datorată probabil efectului stressant al aloxanului (2), (8), se instalează o scădere a activității, care este minimă la 12 ore, urmată din nou de o creștere a ei reflectată în sporul greutății suprarenalelor (sau IPSR). Curba descrisă se încadrează credem în răspunsul general al suprarenalelor la agresiuni.

[1] Șneer, Brătianu, Stroia, Herșcovici, *Modificări morfopatologice corticosuprarenale sub influența administrării aloxanului* (sub tipar).

Din rezultatele noastre observăm că ascorbinemia, cu excepția unei scăderi de 28% față de valoarea normală, pe care am găsit-o ca și Siliprandi (24) în prima oră după injectarea aloxanului, nu numai că nu scade, dar, dimpotrivă, crește la valori de peste 2 600%.

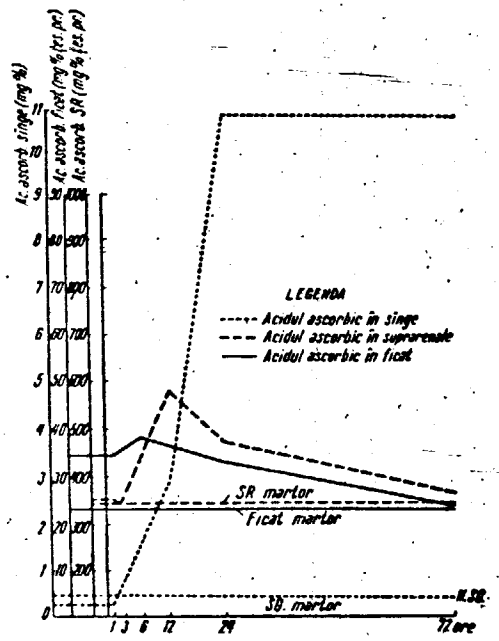

Fig. 3. — Modificările acidului ascorbic din sînge, suprarenale și ficat după administrarea aloxanului (200 mg/kg corp; valori medii).

Nu cunoaștem date privind variațiile vitaminei C în ficat. În condițiile experiențelor noastre, acidul ascorbic a crescut între o oră și 24 de ore, revenind la valori normale după 72 de ore de la injectarea aloxanului (fig. 3).

Faptul ar putea fi interpretat ca o reacție de adaptare la acțiunea toxicului, fie prin depozitarea temporară a unei cantități crescute de vitamină C în ficat, fie consecutiv unei sinteze locale mai ridicate de acid ascorbic sau chiar prin ambele mecanisme.

În ceea ce privește conținutul suprarenalei în acid ascorbic, datele din literatură semnalează toate variațiile posibile, creșteri, scăderi sau lipsa oricăror modificări determinate de aloxan (13).

S-ar putea ca divergențele să țină seamă de faptul că aprecierea s-a făcut în special prin metode histochimice, dar, după cum am menționat, acestea nu ne-au permis să distingem modificările cantitative pe care le-au evidențiat dozările biochimice.

Determinările biochimice ne-au arătat că conținutul în acid ascorbic al suprarenalelor suferă variații în timp, dar niciodată nu a fost găsită o cantitate mai mică decît la animalele de control. Dimpotrivă, am putut constata chiar o creștere cu 60 și, respectiv, cu 40% a acestor valori la

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12 și 24 de ore după injectarea aloxanului în doză mare, pentru ca la 72 de ore să asistăm la o revenire la valorile normale. În cazul dozei mici, creșterea cantității de vitamină C de la nivelul suprarenalelor se menține constantă între 12 și 72 de ore inclusiv.

Din datele noastre rezultă deci că în producerea stressului la animalele aloxanizate în nici un caz nu poate fi incriminată o scădere marcată a acidului ascorbic din sînge, deoarece, cu excepția unei valori minime din prima oră, în toate cazurile ascorbinemia a fost net crescută.

Totuși nu s-a explicat de ce injectarea vitaminei C după 24 de ore de la administrarea aloxanului previne, așa cum arată Chatterjee (3), hipertrofia suprarenalei găsită și de noi după 72 de ore. De altfel într-o altă serie de cercetări am constatat că injectarea vitaminei C în doză mare pare să stimuleze activitatea corticosuprarenală (27).

Faptul remarcat de Devecerski și Frawley (8) că la șobolanii hipofizectomizați aloxanul nu provoacă hiperactivitatea corticosuprarenalei sugerează că substanța nu acționează direct asupra suprarenalei.

Dacă la aceasta adăugăm și observațiile lui Saba și Hoet (11), (23), care, blocînd hipofiza cu fluorocortizol, reduc leziunile pancreatice și împiedică apariția hiperglicemiei, precum și creșterea steroidogenezei produsă de aloxan, putem admite că printre tulburările determinate de acest toxic se pot nota și acelea exercitate de acțiunea substanței prin intermediul axei hipofizo-corticosuprarenalei.

Modificările ponderale deosebite ale suprarenalelor constatate la cele două grupe de șobolani injectați cu doză mică sau mare de aloxan credem că aduc încă un argument în sprijinul celor afirmate mai sus.

Faptul că în condițiile descărcării ACTH vitamina C de la nivelul suprarenalelor nu scade, așa cum ne-am fi așteptat, s-ar putea explica prin aceea că sîngele care irigă glanda are un conținut crescut în acid ascorbic (după cum arată experiențele noastre) sau prin unele modificări de transport (18). În felul acesta am putea înțelege și diferențele dintre rezultatele noastre, în care aloxanul nu a determinat depleția acidului ascorbic, și cercetările efectuate de P. Derevenco și colab. (7), care au studiat pe larg activitatea hipofizo-corticosuprarenală în numeroase condiții de stress farmacologic.

În fine, ar mai trebui să găsim o explicație a cauzelor care determină creșterea cantităților de vitamină C de la nivelul sîngelui.

S-ar putea ca aloxanul însuși sau unii metaboliți rezultați din acțiunea sa asupra țesuturilor să se comporte ca substanțe reducătoare asemănătoare acidului ascorbic. Atît faptul însă că după o oră de la injectarea aloxanului cantitatea de acid ascorbic din suprarenale nu se modifică, iar cea din sînge chiar scade, cît și tehnica de dozare utilizată de noi fac puțin probabilă supoziția emisă mai sus.

S-ar putea ca aloxanul — și aceasta dacă se va dovedi ni se pare mai interesant — să stimuleze sinteza vitaminei C. Cum cercetările noastre s-au efectuat pe șobolani care știm că sintetizează acidul ascorbic ar face mai probabilă această ipoteză.

În acest sens am putea înțelege și modificările observate în prima oră după administrarea aloxanului. Astfel, în timp ce vitamina C din sînge scade, iar cea din suprarenale nu se modifică, conținutul ficatului în acid

ascorbic crește foarte mult (fig. 3). Or, după cum am mai arătat (27), sinteza și depozitarea vitaminei C s-ar face în special la nivelul ficatului.

Numeroasele probleme încă insuficient clarificate, contradicțiile care încă persistă, precum și importanța pe care suprarenala o are în general în activitatea metabolică a organismului și desigur și în tulburările din cadrul diabetului justifică și sugerează necesitatea de noi cercetări.

CONCLUZII

Aloxanul administrat în doze de 100 și de 200 mg/kg corp a provocat modificări deosebite. Sub influența dozei mari, vitamina C din sînge, după ce marchează o ușoară scădere în prima oră, crește foarte mult (12—72 de ore). Doza mică determină o creștere mai puțin marcată.

Sub influența dozei mari, conținutul în acid ascorbic al suprarenalei atinge maximul la 12 ore, după care are tendința de a reveni lent la valorile normale. Doza mică produce o creștere evidentă, care se menține în toată perioada experimentală.

Conținutul în acid ascorbic al ficatului crește chiar din prima oră și revine la normal după 72 de ore (doza mare).

Suprarenala prezintă modificări care atestă descărcarea ACTH endogen, caracterizate prin creștere ponderală, aspecte de fascicularizare și scădere a lipidelor de la nivelul corticalei.

Laboratorul de fiziopatologie, I.M.F. — Iași.

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Modifications du contenu en acide ascorbique du sang, des glandes surrénales et du foie, provoquées par l'administration de l'alloxane chez les rats

RÉSUMÉ

On a poursuivi à différents intervalles de temps (1 à 72 heures), les variations pondérales des glandes surrénales et les modifications de la glycémie, de l'ascorbinémie et du contenu en acide ascorbique des surrénales et du foie, provoquées par l'administration d'alloxane en doses de 100 et de 200 mg/kg de poids corporel chez les rats albinos.

La dose de 200 mg a provoqué, après une faible diminution pendant la première heure, une augmentation très marquée de l'ascorbinémie (12 à 72 heures). La dose de 100 mg a exercé un effet moins marqué.

En ce qui concerne les surrénales, la dose forte a provoqué une augmentation du contenu en acide ascorbique atteignant un maximum après 12 heures et montrant par la suite une tendance à revenir au niveau normal. La dose plus petite a provoqué aussi une augmentation notable et persistante pendant toute la période expérimentale. Dans le foie cette augmentation a été constatée après la dose forte d'alloxane, et suivie d'un retour à la concentration initiale après 72 heures.

Ces modifications qui ont été proportionnelles aux doses d'alloxane suggèrent une éventuelle action stressante diencéphalo-hypophyso-corticosurrénale de cette substance.

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THE PLASMA ASCORBIC ACID OF INFANTS AND CHILDREN

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THE discovery of chemical methods for the estimation of ascorbic acid in the blood gave rise to the expectation that this measurement would be of value in the diagnosis of scurvy and latent forms of this disease. Abt and his co-workers[1] stated, "Such a determination accurately indicates the nutritional state of the body relative to vitamin C." Ingalls[2] reported plasma ascorbic acid figures which, based on his experience to date, indicate the state of vitamin C nutrition. He suggested that 0.5 to 2.0 mg. per cent of plasma ascorbic acid is an optimum level; 0.3 to 0.5 mg. per cent is suboptimum; 0.15 to 0.3, asymptomatic scurvy; and 0.0 to 0.15, scurvy. Certain cases with no clinical or x-ray evidence of scurvy were found by us to have no measurable plasma ascorbic acid. We subsequently made a series of measurements of the plasma ascorbic acid in infants and children and later administered ascorbic acid by mouth to determine its effect on the plasma ascorbic acid.

The method for the determination of the ascorbic acid was that described by Jackson.[3] Five cubic centimeters of blood was obtained by venepuncture and put in tubes containing 2 drops of 20 per cent potassium oxalate and 1 drop of 5 per cent sodium cyanide. The cyanide used has been tested and does not change the reading. It is essential that the blood be not hemolyzed. It was observed that excessive suction on the syringe in obtaining the blood or pressure in transferring the blood to the tube from the syringe were the two main causes of hemolysis. In particular the needle should be taken off the syringe when the blood is blown out. The blood was chilled immediately and kept cold until the plasma was separated; separation was complete very shortly after the blood was obtained. The estimation was made on the plasma with the Evelyn photoelectric colorimeter, using dichlorphenol-indophenol.

Plasma ascorbic acid measurements were made on 158 infants and 77 older children shortly after admission to the hospital.

Chart 1 shows the results obtained on 31 breast-fed infants. It is observed that there is no essential difference between the infants receiving orange juice supplement and those fed on breast milk alone. The average of the whole group is .76 mg. per cent. A few of the infants were suffering from infection, and the measurements of this group were of the same magnitude as the whole group.

From the Department of Pediatrics, University of Toronto, and the Hospital for Sick Children, under the direction of Alan Brown, M.D., F.R.C.P.(C).
Read at the annual meeting of the Canadian Society for the Study of Diseases of Children, Alexandria Bay, N. Y., June 16 and 17, 1939.

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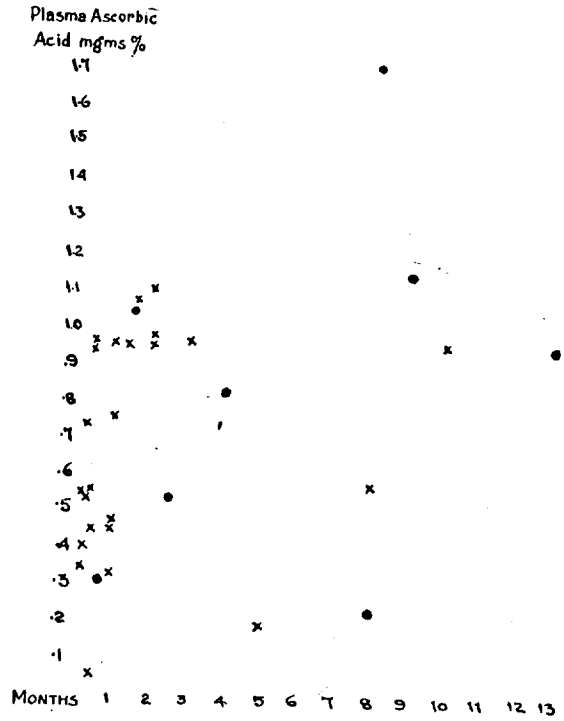

Chart 1.—Milligrams of ascorbic acid per 100 c.c. of plasma in breast-fed infants. (● Indicates supplementary orange juice.)

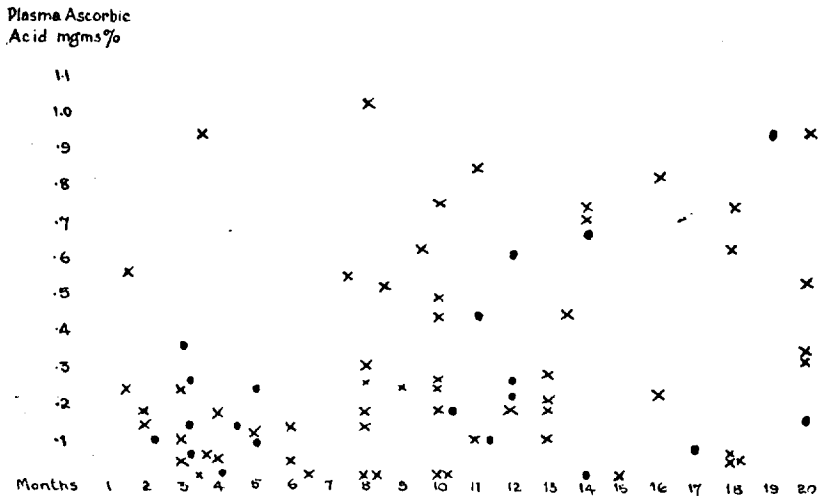

Chart 2.—Milligrams of ascorbic acid per 100 c.c. of plasma in artificially fed infants previously given supplements of 2 oz. or more of orange juice daily (● indicates infants with no infection.)

Chart 2 shows the plasma ascorbic acid measurements on the group of 79 artificially fed infants receiving 2 oz. or more of orange juice daily before admission. There is no difference between the group suffering from infection and those who were free. The average of this group is 0.30 mg. per cent. Practically one-third of this group have measurements of 0.1 mg. or less.

The 48 artificially fed infants receiving little or no orange juice supplement (Chart 3), have an average of 0.12 mg. per cent. Included in this group are 3 infants with scurvy. Two-thirds of this group have measurements of 0.1 mg. or less.

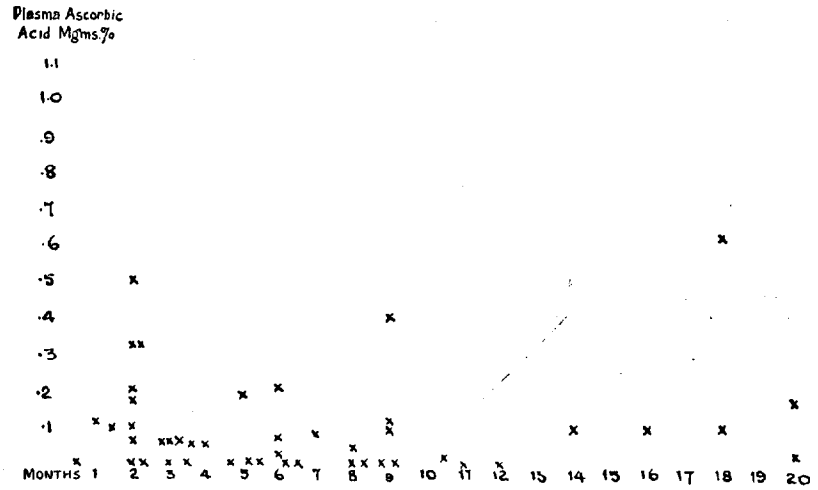

Chart 3.—Milligrams of ascorbic acid per 100 c.c. of plasma in artificially fed infants who had received little, or no, orange juice.

Of the 158 infants, 58 had readings of 0.10 mg. per cent or less, and, of these, only 3 had clinical evidence of scurvy. It is inconceivable, with such a large number of low values, that any interpretation may be made from a low plasma ascorbic acid measurement regarding the presence or absence of scurvy.

TABLE I

AVERAGE PLASMA ASCORBIC ACID ON PATIENTS SHORTLY AFTER ADMISSION TO HOSPITAL

INFANTS			
CLASSIFICATION	TOTAL	RECEIVING ORANGE JUICE REGULARLY	PLASMA ASCORBIC ACID AVERAGE IN MG. %
Breast-fed	31	8	0.76
Artificially fed	127	79	0.23
Artificially fed, no infection	32	20	0.18
Artificially fed, with infection	95	59	0.23
Artificially fed, regular orange juice	79	79	0.30
Artificially fed, no orange juice*	48		0.12

*Three of these had clinical scurvy.

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In summary, 31 breast-fed infants had a plasma ascorbic acid average of 0.76 mg. per cent (Table 1); 127 artificially fed infants had an average of 0.23. Of these artificially fed infants, 79 were receiving 2 oz. or more of orange juice supplement daily and had an average plasma ascorbic acid of 0.30 mg. per cent. Forty-eight of the artificially fed infants with little or no orange juice supplement had an average plasma ascorbic acid of 0.12 mg. per cent.

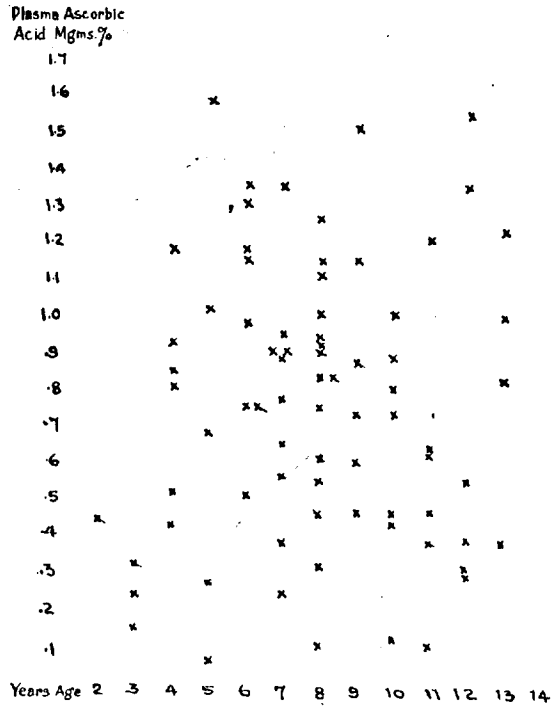

Chart 4.—Milligrams of ascorbic acid per 100 c.c. of plasma in children 2 to 14 years of age on admission to hospital.

Seventy-seven children from 2 to 14 years of age (Chart 4) on admission had an average plasma ascorbic acid of 0.75 mg. per cent. This is higher than that of the artificially fed group and of the same

TABLE II

AVERAGE PLASMA ASCORBIC ACID ON PATIENTS SHORTLY AFTER ADMISSION TO HOSPITAL

CHILDREN, 2 TO 14 YEARS OF AGE		
DIAGNOSIS	NUMBER	AVERAGE PLASMA ASCORBIC ACID MG. %
Respiratory infection	24	0.55
Upper respiratory infection	11	0.79
Rheumatic infection	15	0.87
Renal disease	10	0.73
Diabetes	8	0.96
Miscellaneous	9	0.87
Total	77	0.75

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magnitude as that of the breast-fed infants. Table II shows the relation to disease. There seems to be a tendency to lower values with respiratory infection.

Having found such a large number of infants with very low plasma ascorbic acid measurements who showed no clinical evidence of scurvy, we studied the effect of giving ascorbic acid by mouth to 39 infants with low initial values. One of these had very mild scurvy.

TABLE III

EFFECT OF ADMINISTRATION BY MOUTH OF ASCORBIC ACID ON PATIENTS WITH LOW PLASMA ASCORBIC ACID MEASUREMENTS

MG. ASCORBIC ACID DAILY	NUMBER OF DAYS	PLASMA ASCORBIC ACID AFTER INGESTION	
		RAISED	NO CHANGE
100	1	2	2
500	1	8	4
1000	1	5	3
500	2	3	2
500	3	5	1

Fifteen of 24 patients (Table III) with low initial ascorbic acid and no clinical evidence of scurvy showed a definite rise after receiving one dose of ascorbic acid, and 8 of 11 patients showed a rise after administration over a period of two or three days. Examples of the results are shown in Chart 5.

In Chart 6 are shown plasma ascorbic acid curves of four patients receiving ascorbic acid until a definite rise was obtained. One of these was a mild case of scurvy. It is observed that some patients with no clinical or x-ray evidence of scurvy may require a large amount of ascorbic acid to bring the blood level up.

The patient with scurvy (Chart 6) had a very mild form of the disease. The clinical symptoms had disappeared in seven days, but the ascorbic acid measurement at the end of a ten-day period during which 100 mg. was administered daily by mouth was still zero. In all, 3500 mg. were required to bring this patient up to 1.77 mg. per cent. Another patient with severe scurvy showing a definite laying down of bone in the stripped up periosteum over the hemorrhage had an ascorbic acid measurement of only 0.06 mg. per cent at the time when this was first evident.

DISCUSSION

The finding of higher levels in breast-fed infants than in the artificially fed receiving 2 oz. or more of orange juice as a supplement is interesting. Calculating from the reported figures on pooled breast milk,[4] the average breast-fed baby receives approximately 35 mg. daily, and 2 oz. of orange juice equals approximately 30 mg., so that there is not a great difference between the two. Possible reasons are

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that breast milk enhances the absorption of vitamin C or that the continuous administration throughout the whole day allows for more complete absorption.

Bakwin[5] has recently reported that the artificially fed infant requires from 75 to 100 mg. of vitamin C daily to get the same blood level as the breast-fed infant. He feels that repeated small doses of vitamin C have a much greater effect than one large dose. He also showed the average vitamin C in the blood serum of infants without infection to be 0.34 mg. per cent.

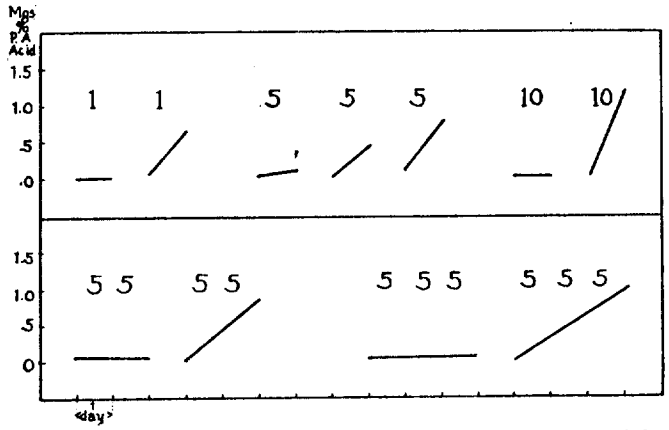

Chart 5.—Examples of ascorbic acid measurements before and 20-24 hours after ingestion of ascorbic acid. (1 = 100 mg. ascorbic acid per day administered by mouth; 5 = 500 mg.; 10 = 1,000 mg.; 15 = 1,500 mg.)

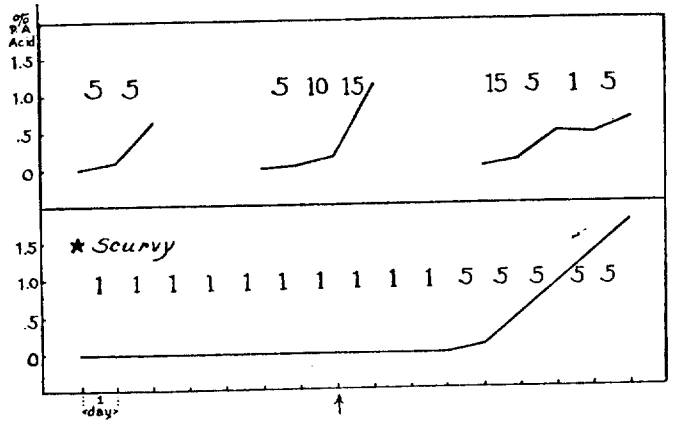

Chart 6.—Records of four patients receiving daily supplements of ascorbic acid until a rise was obtained. (★ indicates a patient with mild scurvy.) (1 = 100 mg. ascorbic acid per day administered by mouth; 5 = 500 mg.; 10 = 1,000 mg.; 15 = 1,500 mg.; ↑, point at which clinical signs and symptoms disappeared.)

The numerous low plasma ascorbic acid values show that there is no justification in the use of this measurement for the diagnosis of scurvy, particularly when the values were obtained in many instances from infants who received orange juice regularly and who had no

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clinical manifestations of this disease. A low plasma value is not a sure indication of generalized body depletion of vitamin C and is of no use as a sole criterion for the diagnosis of scurvy. Kajdi and his co-workers[6] recently reported findings to indicate the unreliability of interpreting low blood ascorbic acid measurements by themselves. He reported a tolerance test in which the curve of absorption is followed in the plasma after an intramuscular injection of ascorbic acid.

In support of this assumption that a low measurement does not indicate the state of vitamin C store in the body as a whole, are the results that we obtained by giving ascorbic acid by mouth. The fact that there was a rise in the plasma ascorbic acid after one administration in many of the subjects suggests that the depots in the body were not entirely lacking in ascorbic acid. One patient with very mild scurvy required 3500 mg. (Chart 6) to bring the blood ascorbic acid level up appreciably. In this patient the clinical symptoms and signs had disappeared with the plasma ascorbic acid still at zero. One patient with acute severe scurvy and large hematomas had laying down of bone in the stripped up periosteum, with a blood ascorbic acid measurement of 0.06 mg. per cent. Obviously these patients were undergoing healing, but this was not reflected in the plasma until considerably more ascorbic acid had been administered.

CONCLUSIONS

Breast-fed infants have higher plasma ascorbic acid measurements than artificially fed babies receiving 2 oz. of orange juice regularly. The latter have higher levels than the patients not receiving orange juice or similar vitamin C supplement. Many artificially fed infants have very low plasma ascorbic acid measurements with no evidence of clinical scurvy.

Low plasma ascorbic acid measurements by themselves do not give one the picture of the general ascorbic acid nutrition of the individual and cannot be used as a standard for the diagnosis of scurvy.

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Cell regenerators, liver
Hepatic damage
Vitamin E
Vitamin C
Nicotinic acid
Belladonna alkaloids
CCl_4

Vitamin E, Nicotinic Acid, Vitamin C and Total Belladonna Alkaloids as Liver Cell Regenerators

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Introduction

Many hazards have been encountered both in animals and man from the use of carbon tetrachloride (CCl_4) in agricultural, industrial and medical fields. Vitamin E, nicotinic acid, vitamin C and total belladonna alkaloids were successfully used by *Soliman* [11] for the prophylactic treatment of hepatic damage induced experimentally in mice by CCl_4. The aim of the present work was to evaluate the effect of the afore-mentioned drugs on mitosis and regeneration in the liver and to test the correlation between cytological and histopathological findings.

Methods

For each treatment 30 three-month-old Swiss albino mice of both sexes were used.

All mice were killed 72 h after the subcutaneous injection of CCl_4 by pithing. Sections were stained with haematoxylin and eosin. Best's carmine, periodic acid Schiff reagents and Feulgen stain were also used.

From every group, specimens from 10 animals were examined microscopically. In order to determine the mitotic index 3,000 cells were counted and the number of dividing cells recorded. In addition, the number of binucleate cells occurring in approximately 600 cells was recorded.

Statistical analysis was carried out according to the method of *Duncan* [2]. The correlation between dividing and binucleate cells was estimated following the methods of *Steel* and *Torri* [12].

Results

Mitotic indexes are recorded in table I. As the distribution of the data is not normal, the values were normalized to

$$\sqrt{0.5 + \text{individual value}}.$$

Analysis of variance of the normalized data showed that the treatments had a highly significant effect on the number of mitosis.

Livers of untreated animals were normal and mitotic divisions were absent.

When a sublethal dose of CCl_4 (4 ml/kg) was administered, microscopic examination revealed central and midzonal hydropic and fatty degeneration or haemorrhagic focal necrosis. The foci

TABLE I

Number of divisions per 3,000 liver cells of mice dosed by CCl_4 only or with different drugs

No.	Blank	Sublethal CCl_4	Control CCl_4 LD_{10}	Vitamin E 50 mg/mouse	Vitamin E 100 mg/mouse	Nicotinic acid 50 mg/mouse	Ascorbic acid 30 mg/mouse	Total belladonna alkaloids 0.05 mg/mouse
1		38			5	31	67	21
2		12		1	–	4	3	37
3		39			7	–	27	28
4		7			1	10	13	42
5		16			4	10	51	37
6		53			25	–	4	63
7		3			1	45	8	28
8		50			2	3	30	11
9		11			1	73	73	49
10		12			3	4	46	59
Total	0	240	0	1	49	180	322	373
Mean	0		0	0.1	4.9	18.0	32.2	37.3

P = 16.95 0.01

N.B.: The difference between any two means not connected by a line is highly significant according to Duncan's multiple range test.

of necrosis were bordered by regenerative cells (fig. 1). Necrosis was focal and so limited that mitosis could take place.

When CCl_4 LD_{10} (8 ml/kg) was administered, the degenerative changes were more advanced. Some lobules showed hydropic degeneration affecting most of the cells. The areas of necrosis were more extensive both in size and in number. Mitotic figures were absent.

Vitamin E in a dose of 50 mg per injected mouse 3 days prior to CCl_4 LD_{10} lessened to some extent the degenerative, fatty and hydropic changes of the liver produced by CCl_4 LD_{10}. The massive necrosis present in the control was reduced to focal in 60% of the animals. Mitotic divisions were absent.

A dose of 100 mg vitamin E per mouse injected 3 days prior to CCl_4 LD_{10} prevented fatty changes and reduced focal necrosis both in size and extent. Some mitotic divisions were observed, but their number was not significant statistically.

Nicotinic acid (50 mg/mouse injected 3 days prior to CCl_4 LD_{10}) checked the degenerative and fatty changes. Limited foci of necrosis were bordered by a few swollen cells with either one large or two normal nuclei. In most of the liver lobules regenerative cells were present. Mitosis was stimulated significantly.

Vitamin C (30 mg/mouse injected 30 min before CCl_4 LD_{10}) greatly reduced the degenerative changes and necrosis and increased the number of regenerative cells. Mitotic figures were abundant.

Total belladonna alkaloids (0.05 mg/mouse injected 30 min before CCl_4 LD_{10}) minimized the degenerative changes and lessened the necrotic foci to a high degree. The regenerative power of liver cells was much stimulated. The regenerative cells were large in size and abundant in number, filling most of the fields (fig. 2). Mitosis was most frequent in this group.

The number of binucleate cells per 600 liver cells of untreated mice and those dosed by CCl_4 alone or in combination with one of the tested drugs are listed in table II. Statistical analysis showed that the differences between treatments were not significant. The correlation coefficient between the number of dividing cells and binucleate cells was computed, but proved to be not significant ($r = 0.028$).

In all slides where mitotic divisions occurred chromosomal abnormalities were observed. These consisted of dicentric chromo-

Fig. 1. CCl_4 sublethal dose. Central and midzonal necrosis of liver is bordered by many regenerative cells. H. and E.: × 230.
Fig. 2. CCl_4 LD_{10} total belladonna alkaloids 0.05 mg minimal foci of necrosis and abundant regenerative cells. H. and E.: × 230.

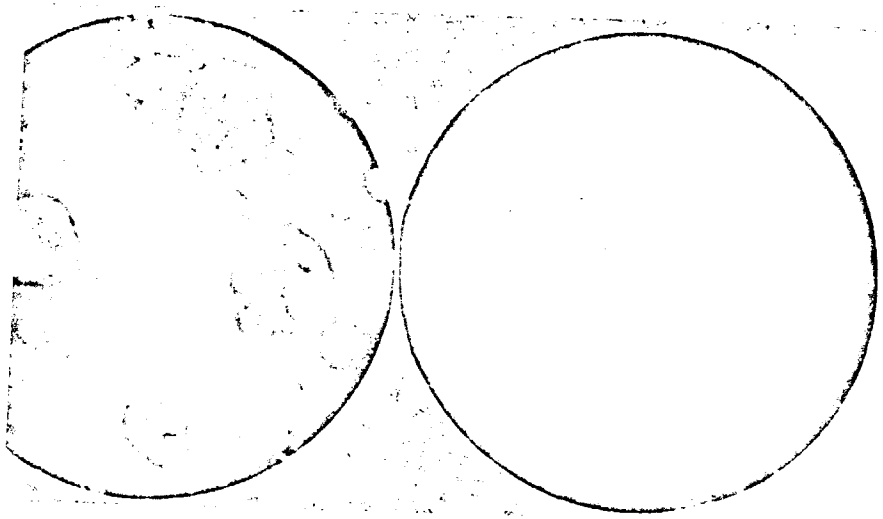

Fig. 3. Bridge connecting telophase nuclei. H. and E.: × 1920.
Fig. 4. Pole chromosomes approaching metaphase plate. H. and E.: × 1920.

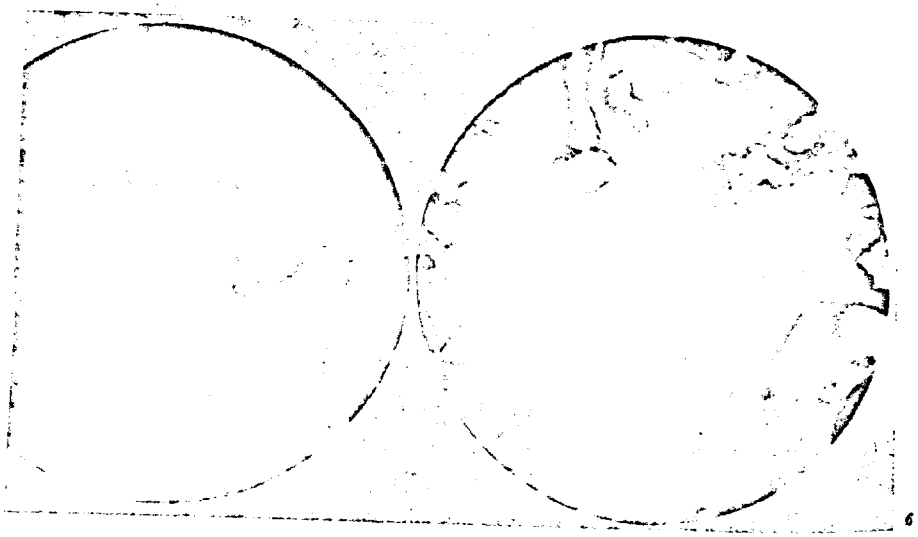

Fig. 5. Three groups metaphase. H. and E.: × 1920.
Fig. 6. Multipolar spindles. H. and E.: × 1920.

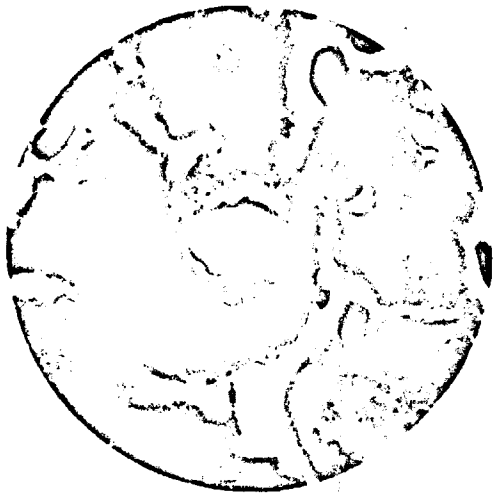

Fig. 7. Early star metaphase. H. and E.: × 1920.

TABLE II

Number of binucleates per 600 liver cells of untreated mice dosed by CCl_4, either alone or in association with different drugs

Treatment	Animal No.											
	1	2	3	4	5	6	7	8	9	10	Total	Mean
Normal	29	89	81	78	110	100	96	95	94	92	864	86.4
CCl_4 (LD_{10}) + Vit. E 50 mg	75	47	47	67	55	61	58	59	132	72	673	67.3
CCl_4 (LD_{10}) + Total Bell. Alkal. 0.05 mg	69	60	58	81	60	66	54	68	66	27	609	60.9
CCl_4 (LD_{10}) + 50 mg Nicotinic acid	64	49	59	89	54	54	59	69	32	62	591	59.1
CCl_4 (LD_{10}) (8 ml/kg body)	62	46	58	60	66	68	48	59	58	58	583	58.3
CCl_4 (LD_{10}) + Vit. E 100 mg	27	66	48	36	48	63	61	55	64	48	516	51.6
CCl_4 (LD_{10}) + 30 mg Vitamin C	58	95	19	35	62	58	59	28	23	60	497	49.7
CCl_4 sublethal (4 ml/kg body)	39	47	46	36	62	57	36	23	50	64	460	46.0

$P = 0.636$ (0.05 No significant difference exists between different groups).

somes (fig. 3), pole chromosomes (fig. 4), three group metaphases (fig. 5, multipolar spindles (fig. 6) and star metaphases (fig. 7).

Discussion

Mitotic divisions were absent in the livers of the blanks. The scarcity of dividing cells in the adult liver has been reported frequently.

No mitotic divisions were observed in controls or those treated with vitamin E, in spite of the mild prophylactic effect of the latter clinically and histologically [3].

The highly significant effect of nicotinic acid, ascorbic acid and total belladonna alkaloids on the mitotic index proved that these drugs are good liver cell regenerators. This confirms the previous results of *Soliman* [11].

Dividing cells occurred mainly in the vicinity of the central vein. Quite often they were seen in the immediate neighbourhood of necrotic cells.

Many binucleate cells were observed. Since they have been reported as occurring frequently in regenerating tissue [1], the possibility could not be overlooked that their percentage would be affected by the various treatments. However, statistical analysis proved that the treatments did not cause significant differences in the number of binucleate cells. Moreover, there was no correlation between the number of binucleate cells and of dividing cells.

It may, therefore, be concluded that:

1. the frequency of binucleate cells varies between animals receiving identical treatment due to causes as yet unknown;
2. the frequency of binucleate cells is not related to mitotic activity;
3. in the present material regeneration was not accompanied by binucleate cell formation.

The chromosomal abnormalities were probably due to a residual effect of CCl_4, as examination of the livers of animals that received a sublethal dose of CCl_4 only, showed abnormalities similar to those found in animals receiving other treatments.

Dicentric chromosomes are a result of chromosome breakage followed by differential reunion. They have been reported as a result of radiation [4, 13], and of treatment with various chemicals

[5]. In the present material they were usually broken by cell wall formation, but sometimes they were seen to connect the two daughter cells.

Pole chromosomes, three group metaphases and multipolar spindles are also described by *Siebs* [10] as a result of treatment with various chemicals, especially carcinogenic hydrocarbons and diethylstilboesterol. They are caused by a disturbance in the prometaphase movement, which is thought to be controlled by permanent fibres connecting each centromere to a specific centriolar region [7]. The action of the central spindle, which governs anaphase movement, is not affected.

Star metaphases are a well-known result of treatment with colchicine. Metaphase is arrested, the chromosomes are overcontracted and anaphase does not occur due to a disturbance in the central spindle. The chromosomes become clumped, and ultimately the cell dies.

Although the numbers of mitotic figures observed do not permit a statistical analysis of the percentage and distribution of the abnormalities, it would appear that star metaphases were somewhat more frequent in the material treated with CCl_4 only and also where vitamin E is administered, which might indicate a protective effect of nicotinic acid, total belladonna alkaloids and vitamin C against disturbance of the central spindle.

In conclusion, it may be said that CCl_4 has a profound effect on the cell nucleus, causing chromosome breakage and disturbances of the central as well as the permanent spindle. As ATP is related to spindle formation, the observed irregularities of the spindle are in accordance with the reports concerning the decrease of liver ATP due to CCl_4 [6, 8, 9].

Summary

Experimental liver damage was induced in mice by subcutaneous injection of CCl_4 (LD_{10}, 8 ml/kg). The protective effect of vitamin E, nicotinic acid, vitamin C and total belladonna alkaloids against liver injury was studied from the histopathological and cytological point of view.

The prophylactic action of the drugs was lowest in the case of vitamin E, where degenerative changes were lessened but regenerative or dividing cells were very few.

Nicotinic acid, vitamin C and total belladonna alkaloids showed increased prophylactic effect in that order. Reduction of degenerative changes was accompanied by a simultaneous increase in the number of regenerative and dividing cells.

The number of binucleate liver cells was not affected by the various treatments and therefore, not related to mitotic activity and regeneration.

CCl_4 caused mitotic irregularities which could be attributed to chromosome breakage and spindle disturbances.

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Lognormal Model for Ascorbic Acid Requirements in Man

Edward L. Spitznagel, Jr.

Results have recently been obtained which call into question the correctness of the minimum daily adult requirement of vitamin C set down by the Food and Drug Administration. In particular, Pauling (1970), Ritzel (1961), and Stone (1967) indicate that large amounts can have a prophylactic effect on the common cold. Also, Gildersleeve (1967), Pauling (1970), and Regnier (1968) indicate that large amounts can abort a cold in its very early stages and suppress the symptoms of a fully developed cold. In experiments with guinea pigs, Williams and Deason (1967) found a twentyfold variation in the amount of vitamin C needed to prevent symptoms of scurvy and concluded there must be at least as much variability in the vitamin C requirement in man. Finally, Bourne (1949), Pauling (1970), and Stone (1966) give entirely distinct arguments that indicate a typical human daily vitamin C requirement for maintenance of full health is on the order of grams, perhaps one to five, rather than milligrams.

A number of studies on the relationship of vitamin C to the common cold have been published. Some of them, notably the large-sample studies of Glazebrook and Thomson (1942), Cowan et al (1942), Ritzel (1961), and Gildersleeve (1967) lend some support to the claim that vitamin C does reduce the incidence and severity of colds. Others, notably those of Tebrock et al. (1956) and Walker et al. (1967), seem to support the opposite conclusion.

The purpose of this paper is to show that the four affirmative studies mentioned above can be made to fit very neatly a log-normal distribution with large variance. As a result, these studies will be seen not to stand alone but to support one another. In addition, the fitted distribution will lend support to the conclusion of Williams and Deason mentioned above and to the computations by Bourne, Pauling, and Stone of the typical daily amounts of vitamin C required for full health.

A random variable is said to have a log-normal distribution LN (μ, σ^2) if its natural logarithm is normally distributed N(μ, σ^2). The content of the central limit theorem is that a random variable which is the sum of many independent (sufficiently well-behaved) random variables is approximately normal and a random variable which is the product of many independent (sufficiently well-behaved) random variables is approximately log normal (Aitchison and Brown, 1957). When σ in LN (μ, σ^2) is considerably less than 1, the log-normal random variable is essentially indistinguishable from a normal one, so certain log-normal random variables can, for simplicity, be treated as normal. This fact may well account for the prevalence of the normal distribution in nature, even though the random variables concerned often seem to be products of independent effects rather than sums. However, when σ is not much less than 1, the distribution LN(μ,σ^2) is easily distinguished from normal by virtue of its striking skewness.

Pauling (1970) and Stone (1967) both suggest that extensive machinery has evolved that enables the human body to survive on a vitamin C intake that is perhaps 5 to 10% of the daily intake for the common ancestor of man and those other primates unable to produce their own supply of ascorbic acid. Since these evolutionary developments had to occur over the few million years during which the diet of man and his immediate predecessors underwent its great changes, it seems likely that there still must be great variability of this machinery within individual people. Also, it seems likely that the various modifications interact in multiplicative fashion. These considerations lead us to propose a log-normal model for the amount of vitamin C required for optimum human health.

Optimum human health, of course, is difficult if not impossible to describe accurately. We can, however, make the following assertion: The common cold is such a difficult disease to prevent or ameliorate that if vitamin C does in certain doses produce cold prevention or relief, then those doses are probably the same order of magnitude as the doses required for optimum human health. And, if a log-normal model can be fitted to the amounts of vitamin C required for cold prevention or relief, then probably a log-normal model is the correct description of the amount of vitamin C required for optimum human health.

In what follows, the results of the four affirmative studies mentioned above, together with a piece of "common knowledge" information will be plotted on normal by logarithmic graph paper. It will be seen that the five points fall remarkably close to a straight line. From the best-fitting line, we will obtain estimates of the parameters μ and σ of the lognormal model. Of course, certain reasonable assumptions will be necessary in order to bring the five different pieces of information onto a common ground. Two of these assumptions are as follows:

I) Dosages of vitamin C have approximately a threshold effect on the common cold; that is, for any one individual there is a dosage level such that doses below this level do little good, while doses above this level are notably effective. Of course, this individual threshold can be expected to vary from day to day depending upon stress, exposure, and other factors, but as long as the within-individual variation is small compared to the between-individual variation, there is no problem in regarding it as fixed—equal, say, either to its mean or median value.

II) In all but one of the five cases to follow, we will take the background vitamin C level from food to be 100 mg/day. Given a good ordinary diet, a person can be expected to receive be-

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tween 100 and 300 mg vitamin C daily from food. However, it seems likely that most people will fall toward the lower end of this range due to current methods of food preparation and eating habits.

We are now ready to derive the five data points plotted in Figure 1. The derivations below are labeled as the points are labeled, A through E.

A. It is known that a certain fraction of people rarely or never catch cold. Pauling (1970) reports this fraction as being somewhere between 6 and 10%. Splitting the difference, we can estimate that the background dosage of 100 mg vitamin C daily is sufficient to produce cold resistance in approximately 8% of the population.

B. Glazebrook and Thomson (1942) performed a study on approximately 1435 adolescents in a boarding school in Scotland. They introduced 200 mg vitamin C per day into the food of each of 335 students and held the rest (1100) as controls. Their tests of the institution's food revealed it to be prepared so poorly that the vitamin C from food was only 10 to 15 mg per student per day. Thus, the amount of vitamin C received by a student in the control group was negligible, while the total amount received by each member of the control group was approximately 200 mg per day. During the period of observation, members of the control group had 286 colds, or 0.260 colds per capita, while members of the vitamin C group had 72 colds, or 0.215 colds per capita. If we use Assumption I above to ascribe this reduction primarily to 200 mg vitamin C per day making a certain fraction of the population resistant to colds, we can estimate that fraction to be

$$1 - 0.215/0.260 = 17.3\%$$

C. Cowan et al. (1942) performed a study on between 363 and 427 students at the University of Minnesota. The students were approximately evenly divided between those receiving vitamin C and those receiving placebos. The students receiving vitamin C took 200 mg per day. By Assumption II, we estimate their total daily dosage to be 300 mg and the total daily dosage of the control students to be 100 mg. During the period of observation, members of the control group had 2.2 colds per capita, while members of the vitamin C group had 1.9 colds per capita, indicating that increasing dosage from 100 to 300 mg daily has the effect of producing cold resistance in

$$1 - 1.9/2.2 = 13.6\%$$

of those not already resistant due to the 100-mg background dosage. Since we have already estimated that 8% of the population is resistant from this background dosage, we can estimate that 300 mg daily dosage produces cold resistance in

$$0.08 + 0.136 \times 0.92 = 20.5\%$$

of the population. In regard to the study quoted, we might note two points of interest. The investigators recognized that the difference in frequency of colds, 1.9 vs. 2.2, was significant at the 0.05 level, but they questioned the practical value of their finding. Pauling (1970) notes that the differences in dropout rates from the study and the differences in days lost from school are also significant, at the 0.01 and 0.001 levels, respectively.

D. Ritzel (1961) performed a double-blind study of colds in 279 skiers. A group of 139 received 1 g vitamin C per day, while the remainder, 140, received a placebo. He reported 42 individual symptoms in the group receiving vitamin C and 119 symptoms in the control group. If we assume that the number of colds is proportional to the number of symptoms, then we can proceed as in part C to calculate that 1100 mg daily dosage of vitamin C produces cold resistance in

$$0.08 + 0.645 \times 0.92 = 67.3\%$$

of the population. We also note that Ritzel reports total days of sickness as 31 vs. 80, from which we can calculate a second estimate, very close to the one above. Ritzel's results all have high statistical significance (0.001 level.)

E. Gildersleeve (1967) studied the effects of 5-g daily dosage of vitamin C on 400 colds in 45 patients over a period of 4 years. His experience was that in 95% of the cases all or nearly all symptoms were suppressed and remained suppressed for the duration of the cold if the dosage was maintained. This information is not strictly comparable with that of the previous studies since it involves cold relief rather than prevention, but it seems to be the only large-sample result for such a high dosage level. We will therefore try to make as best use as possible of it.

Assuming that at large doses the number of colds which can be prevented is approximately the same as the number which can be suppressed, we estimate that 5.1 g vitamin C daily will produce cold resistance in

$$0.08 + 0.95 \times 0.92 = 95.4\%$$

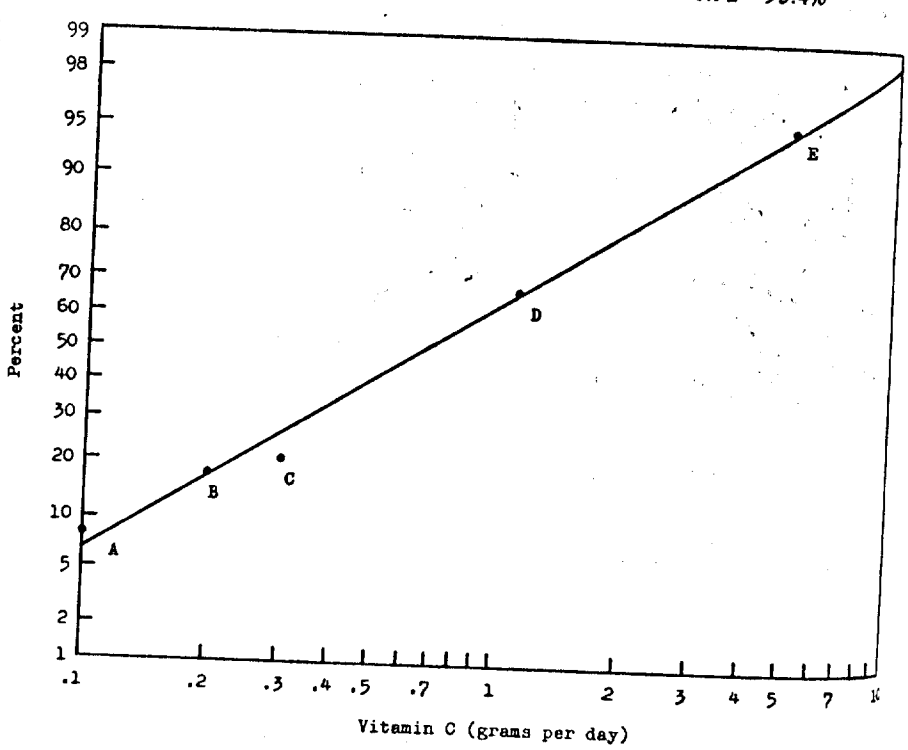

Fig. 1. Percentage of population made resistant to colds by various daily doses of vitamin C.

of the human population. As we will see, this fifth data point fits in very well with the other four, which is encouraging, even though we may not wish to place so much confidence in it. If one accepts Gildersleeve's result but feels that even at large doses the number of colds prevented will be greater than the number of colds whose symptoms can be suppressed, then the plotted point can be interpreted as indicating the right tail of the true distribution is not quite as full as the log-normal tail.

Figure 1 shows the five data points A through E plotted on normal by logarithmic graph paper, together with the least squares regression line (percentile on dose) fitted to them. From the fitted line, we can make the following estimates for our log-normal model:

$$\exp \mu = 650 \text{ mg}$$
$$\sigma = 1.23$$
$$\text{median} = 650 \text{ mg}$$
$$\text{mean} = 1450 \text{ mg}$$
$$\text{lower 2.5 percentile} = 55 \text{ mg}$$
$$\text{upper 2.5 percentile} = 7600 \text{ mg}$$

Our model thus indicates a much greater variability, over a hundredfold, for the vitamin C requirement in humans than the twentyfold variability that Williams and Deason (1967) found for guinea pigs. The question arises whether this great variability is reasonable, especially at the upper end of the scale. Are there 2.5% of human beings whose resistance to colds could be improved by dosages of 7.6 g or more vitamin C per day but by no smaller amount? At present we cannot say. As mentioned above, perhaps the right tail of the model does fall off appreciably faster than the log-normal tail. On the other hand, it has been found (Conney et al., 1961) that rats have the property of increasing their vitamin C production fourfold under stress, the upper limit of their production being extrapolated by Stone (1966) to approximately 15 g per day in a human being. Perhaps, then, there is a small percentage of people with minimal evolutionary adaptations who could profit by such large doses.

In view of the small number of data points obtained, we should make some attempt to assess the reliability of our results. First, let us remark that the choice of studies made here was not after the fact; we seem to have used the full set of large-sample studies amenable to our kind of analysis. The sample size (measured in colds or cold symptoms) must be sufficiently large to assure

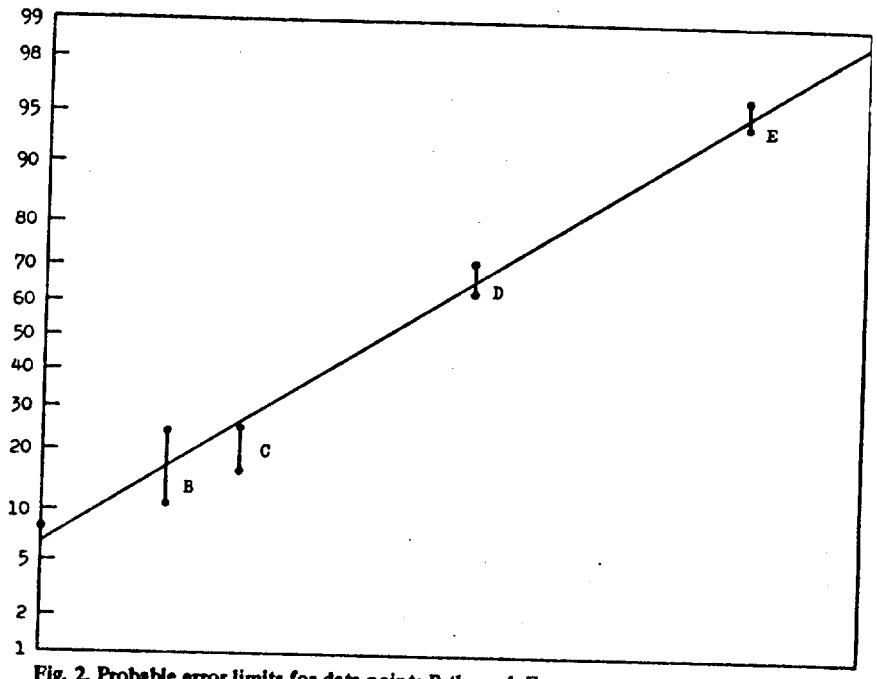

Fig. 2. Probable error limits for data points B through E.

reasonable stability of the data points computed. In Figure 2 we have sketched some very crude probable error (50% confidence) limits on the data points B through E. These were computed from Poisson and binomial models and do not include the additional uncertainties introduced by the error of point A and the random or not-so-random choice of individuals. Nevertheless, these limits seem to represent an appreciable portion of the random error, and as can be seen they are not unreasonably spread apart. Of course, there could be additional errors due to biases in the individual experiments. Each individual must assess this source of error for himself, as it seems impossible to reach any agreement on it.

Passing from estimates of error in the individual data points, we consider the amount of consistency among the five points actually plotted. Visual inspection reveals that they do indeed lie quite close to a single straight line. Beyond this, various parameters were jackknifed to assess their reliability. In general, the results were quite close to those obtained from the single least-squares line. For example, two jackknifed estimates were:

$$\exp \hat{\mu} = 630 \text{ mg}$$
$$\hat{\sigma} = 1.22$$

and 95% confidence limits on the value of μ calculated from the jackknife were 520 and 770 mg.

We come now to two important negative studies mentioned earlier. The one study was conducted by Tebrock et al. (1956) on nearly 2000 patients in three different parts of the country. The dosage was 200 mg/day continued over 3 days, and the study was concerned with whether vitamin C could shorten the duration of an already developed cold. All investigators who claim that vitamin C can alleviate cold symptoms indicate that the amount needed is on the order of 20 to 30 times the amount used in this study. Thus, it appears that the study failed largely through lack of an adequate experimental design. Not having previous experience to guide them, it would have been better for the investigators to have tried a number of different dosage categories in the hopes that some difference would show up in an analysis of variance.

The other study is that of Walker et al. (1967). Their study was on the effect of 3 vitamin C per day on prevention and relief of the common cold. Of 47 dosed subjects and 44 control subjects, 18 in each group caught cold when all 91 volunteers were inoculated with cold viruses on the third day. There seemed to be no difference in severity or duration of the colds under continued dosage. Of course, our model would have predicted a ratio of 3:33 as opposed to the observed ratio 18:18, and these two ratios are too disparate for the latter to have occurred by chance, assuming the model applied. Thus this study appears to furnish strong evidence against our model. There are, of course, certain

reasons why our model may not apply in this case. As Pauling (1970) points out, the inoculations may have introduced a much larger amount of virus particles than would have been introduced by airborne infection; also, the dosage continued over just 2 or so days prior to inoculation may not have been enough to fill the person's depleted stores.

In any event, it does appear that the conclusions of this experiment are in contradiction to the results which we have found fit together rather well into the log-normal model. It would seem that further experimentation is in order to resolve the difference and, if possible, to provide a strong test of the correctness of the model.

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Ascorbic Acid Deficiency Associated with Dilantin Hyperplasia*

by

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CLINICALLY EVIDENT MALNUTRITION is rarely seen in dental patients in the United States. However, recent reports [1] would appear to indicate that many individuals in this country are deficient in one or more of the essential nutrients. This state of malnutrition may be due chiefly to factors secondary to mental, physical and physiological stresses, or often as a result of other pathologic diseases, although studies suggest that social and economic factors could not be ruled out in some cases.[1] These malnourished individuals rarely present easily identifiable clinical manifestations which are pathognomonic of deficiencies of specific nutrients and therefore good judgment is required of the clinician who must initiate essential biochemical tests. This is exemplified by a case of ascorbic acid deficiency recently seen at the Seattle Veteran's Administration Hospital.

Case Report

A 54 year old, black male with left hemiplegia (incurred during a cerebral-vascular accident) was referred to the dental service of the Seattle Veteran's Administration Hospital for evaluation of his oral condition. The patient was receiving medication consisting of Dilantin, 250 mg and phenobarbitol, 25 mg daily.

A review of the patient's record revealed that this was the seventh admission to the Veteran's Hospital. An oral examination performed in 1969 had revealed that the gingival tissues were enlarged and fibrotic, with the exception of the marginal gingiva which was soft, erythematous, and bled easily. Radiographs and probing revealed moderate to advanced periodontal disease. Repeated thorough curettage was performed at that time and no remission of the condition was noted post-curettage. Unfortunately the patient left the hospital against medical advice and no follow-up could be made.

Clinical examination during the most recent admission (1971), revealed a listless, lethargic, black male who apeared at times during the examination to be extremely irritable. His oral hygiene was poor as evidenced by gross accumulation of calculus and plaque. Dilantin hyperplasia was evident; the marginal gingiva was erythematous, swollen, spongy, bled easily, and was covered with fibrin. The attached gingiva was bulbous, fibrotic, and showed regional absence of stippling (Figure 1). Radiographically, there was vertical bone loss, especially around the molar teeth (Figure 2). Other significant findings were mild chronic pain associated with his elbow and knee joints.

Local periodontal therapy consisting of coronal scaling and root curettage was instituted. Eight days later no improvement was noted. Careful consideration was given to the following clinical and laboratory findings: (1) guaic positive stools, (2) irritability, (3) enlarged, erythematous, fibrin-covered marginal and attached gingiva, (4) bleeding tendencies of the gingiva, (5) enlarged, fibrous attached gingiva.

Previous laboratory tests indicated no gross abnormalities except for evidence of borderline anemia. Upper and lower gastro-intestinal series were negative. Biopsy of the marginal gingiva showed edema, hemorrhages, and infiltration by lymphocytes and plasma cells.

These clinical observations and laboratory findings led to the suspicion of ascorbic acid deficiency. Further questioning of the patient revealed that his normal diet consisted of only one bowl of cooked cereal per day. This gave further support to the tentative diagnosis of ascorbic acid deficiency associated with dilantin hyperplasia.

Biochemical Tests

Plasma ascorbic acid level was determined by osazone formation with 2,4-dinitrophenyl hydrazine, which measures both the reduced and oxidized forms of the vitamin after conversion to the oxidized form and 2,3-diketo-L-gulonic acid.[2] The plasma level was 0.08 mg/100 ml which was compatible with marked tissue depletion of the vitamin. No ascorbic acid was detected in a 24 hour urine collection. The patient was given an ascorbic acid load test of 1000 mg per day orally for six days during which time 24 hour urine collections were assayed for ascorbic acid excretion. The results of these assays are seen in Figure 5. This figure depicts the plasma concentration of ascorbic acid on days one and five as well as the daily urinary loss of the vitamin. Plasma level on day five was 0.54 mg/100 ml and the daily urine level showed a gradual increase ranging from

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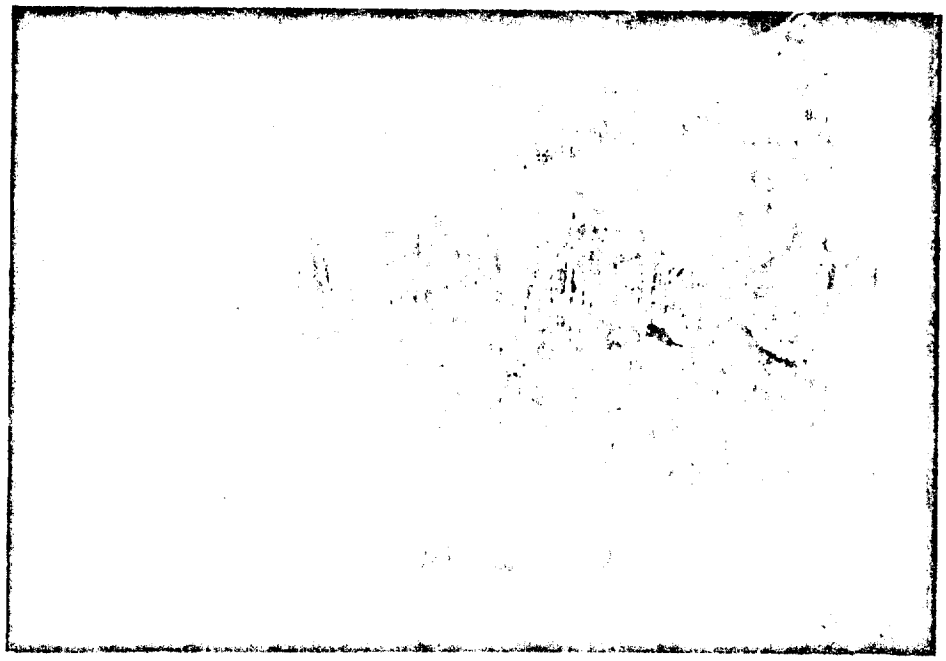

Figure 1

zero on day one to 25.5 mg/100 ml on day five. The increase in the urinary level of ascorbic acid was most prominent after the third day of feeding with the vitamin.

On day six of the load test, it was noticed that the patient's appetite was better and his attitude seemed much improved. The patient's gingival condition was seen to improve somewhat earlier. On day five of the load test, a marked reduction of edema and ease of bleeding was noted and the marginal gingiva appeared less erythematous (Figure 3). The patient's gingival condition on day thirteen of administration of ascorbic acid was markedly improved although no local factors had been eliminated (Figure 4). The patient was given vitamin supplements, general dietary counseling and referred to the hospital dental service for oral prophylaxis, oral hygiene instruction, and definitive dental care.

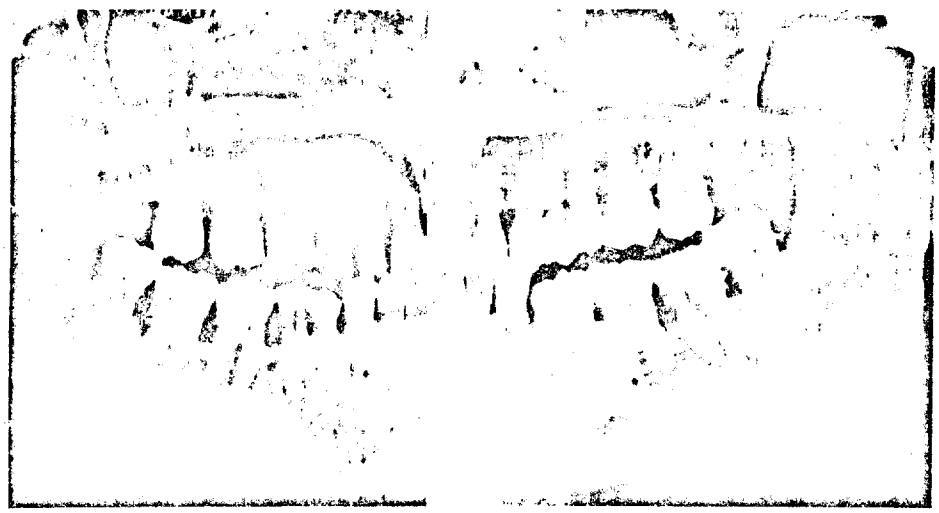

Figure 2

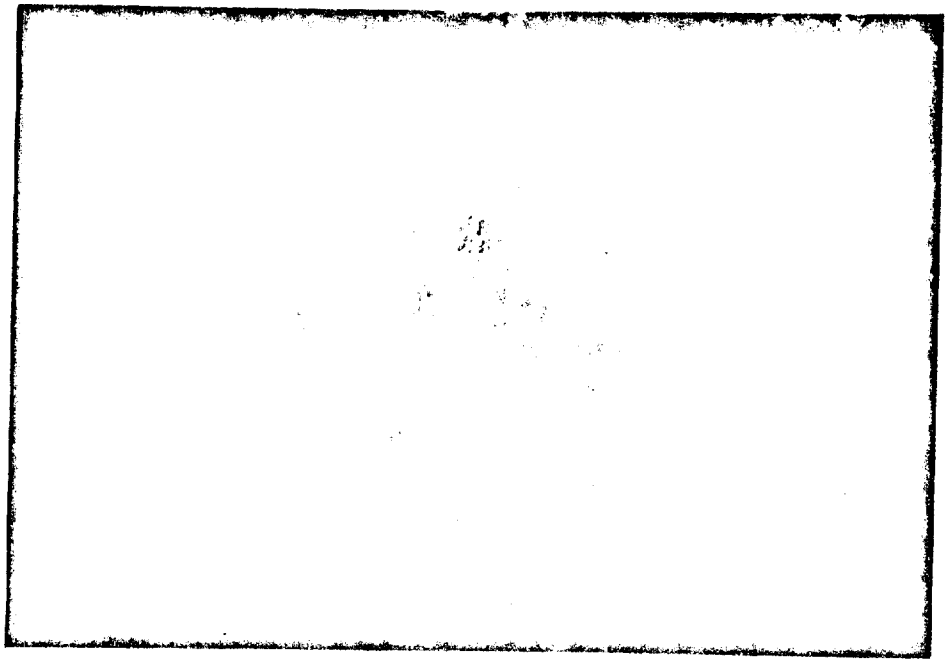

Figure 3

Discussion

The importance of ascorbic acid for the maintenance of good health has been recognized for many years but little is known of its mechanism of action.[3] There is, however, a good body of evidence to indicate that ascorbic acid plays a major role in collagen biosynthesis and maturation. Ascorbic acid also is reported to function as a co-factor in many enzymatic pathways. Recent reports suggest that this vitamin is essential for the maintenance of the basement membrane.[9]

The role of ascorbic acid in oral health and disease state has been controversial. It has been reported that

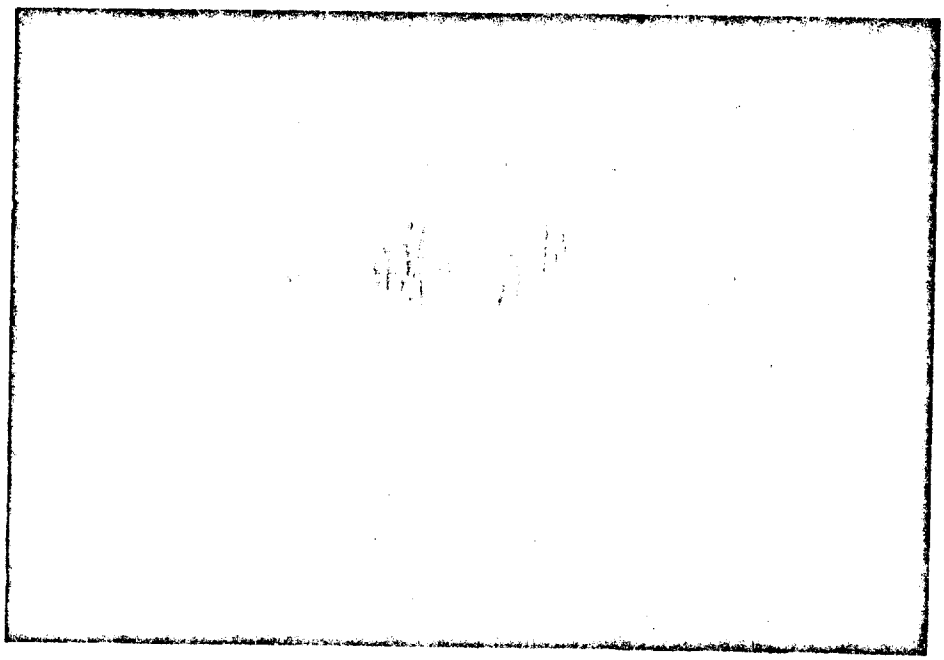

Figure 4

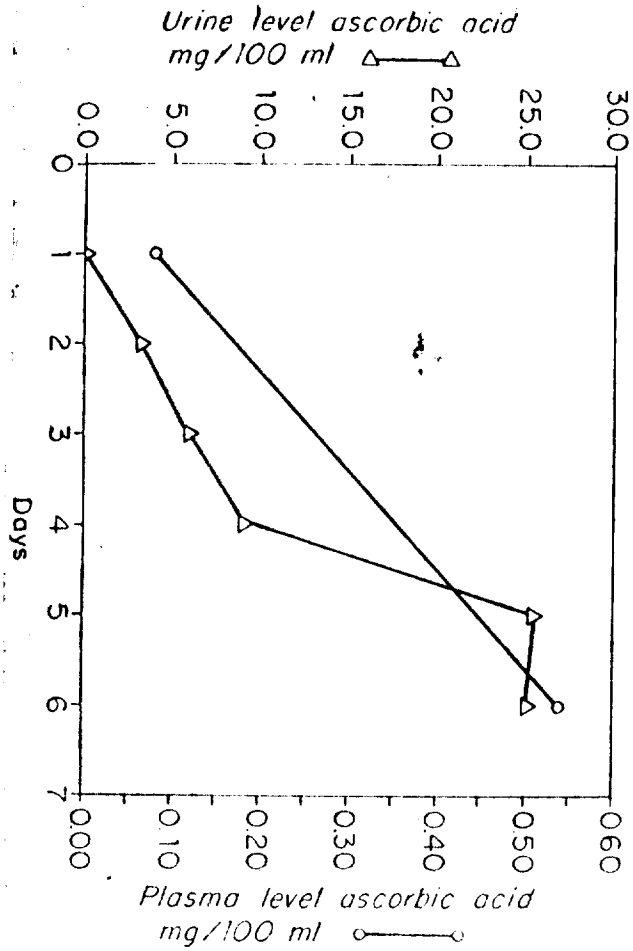

Figure 5

prolonged marginal deficiency of ascorbic acid plays a contributory role in the initiation of periodontal disease.[5,6] However, this view is not generally substantiated by the findings of other investigators.[7,8]

Among the clinical signs of gross ascorbic acid deficiency are petechial hemorrhages, joint effusions, subconjunctival hemorrhage, dyspnea, hyperkeratosis, perifollicular congestion, gingival changes, arthralgia, and femoral neuropathy. The severity of the gingival changes are directly related to the degree of gingivitis and are associated with plasma ascorbic acid levels below 0.16 mg/100 ml and body pool size below 300 mg.[3] However, some of these signs may not be manifest in all cases.

In the case presented, the sole clinical manifestation was the chronic gingival lesion which in itself was not pathognomonic of ascorbic acid deficiency. The patient showed marked diminution in serum vitamin C level and although this parameter is a reflection of the recent intake of the vitamin, there is good evidence that serum ascorbic acid level below 0.2 mg/100 ml is compatible with scurvy.[3,4] The virtual absence of ascorbic acid in the urine when the patient was first seen indicated marked tissue desaturation of the vitamin and this was further confirmed by the very low excretion in the initial phase of the load test. Remission of the oral lesions followed the rise in serum levels of the vitamin.

This case emphasizes the need for adequate consideration of a patient's dietary history in the clinical management of oral lesions.

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The Effect of Cortisone and Adrenocorticotropic Hormone on the Dehydroascorbic Acid of Human Plasma

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(*Received* 30 *May* 1952)

It is well known that ascorbic acid in aqueous solution is easily and reversibly oxidized to dehydroascorbic acid, and it is believed that above pH 4 the latter can undergo irreversible change, possibly to diketogulonic acid, as a preliminary to further oxidation. The possibility of the existence of dehydroascorbic acid or diketogulonic acid in blood plasma, however, has been generally ignored since the time of Borsook, Davenport, Jeffreys & Warner (1937) who, with van Eekelen (1935), Kellie & Zilva (1936), Farmer & Abt (1936) and Ralli & Sherry (1944), claimed that vitamin C exists in plasma in the reduced state as ascorbic acid. The brief statement that dehydroascorbic acid exists in human blood has recently been made by Chen & Schuck (1950) but no supporting evidence was given. Apart from its academic interest, the relation between dehydroascorbic acid and experimental diabetes (Patterson, 1950) and the close association of ascorbic acid with the adrenal cortex and stress (Selye, 1946) suggest that the form in which vitamin C exists in blood is worthy of investigation. The experiments described in this paper establish the facts that a significant part of the total vitamin C present in normal human plasma exists as dehydroascorbic acid, that diketogulonic acid does not exist in plasma in significant quantities, and that the ratio between dehydroascorbic acid and ascorbic acid can be consistently influenced by adrenocortical steroids.

EXPERIMENTAL AND RESULTS

The existence of dehydroascorbic acid in human plasma

Chemical methods. Plasma from normal human subjects was submitted to the technique of Roe & Keuther (1943) and to a procedure employing 2:6-dichlorophenolindophenol. The former involves preliminary oxidation of ascorbic acid with activated charcoal and the colorimetric estimation of the reaction product with 2:4-dinitrophenylhydrazine under defined conditions. It is claimed to estimate the dehydroascorbic acid produced by oxidation of ascorbic acid plus any preformed dehydroascorbic acid and diketogulonic acid (i.e. 'total ascorbic acid'). The procedure involving 2:6-dichlorophenolindophenol was applied to a sample of the same plasma in order to estimate ascorbic acid itself. For this purpose 3·0 ml. of 3% (w/v) metaphosphoric acid were added to 2·0 ml. of plasma, and the precipitated protein separated by centrifugation and filtration. A known excess of standard solution of the indophenol was added to a sample of the filtrate, and the residual colour was measured in a spectrophotometer (Unicam SP 500) at a wavelength of 480 mμ., 30 sec. from the time of addition of the indophenol. The method is similar in principle to that of Mindlin & Butler (1937–8), except that it was found possible to dispense with the buffer, and greater accuracy was obtained by constructing a standard curve instead of using a single standard solution.

The validity of the procedure was first tested by measuring the rate of fading of the indophenol solution under varying conditions, and the results are shown in Fig. 1. The indophenol was decolorized rather slowly in the presence of aqueous metaphosphoric acid (curve *A*) and more rapidly in the presence of trichloroacetic acid (curve *B*) at the same pH. Ascorbic acid added to the metaphosphoric acid solution (curve *C*) completely stabilized the indophenol colour after the initial reduction due to oxidation of the ascorbic acid. Unfortunately, it was impossible to obtain readings earlier than 20 sec. after the addition of the indophenol, but the reduction of the dye by ascorbic acid is certainly completed within 30 sec. When the indophenol was added to a metaphosphoric acid solution containing a trace of H_2S (as an example of a substance oxidized slowly by the indophenol) fading continued to occur after 30 sec. and for as long as determinations were made (curve *D*). A similar slow reduction of the dye occurred in the presence of metaphosphoric acid and $Na_2S_2O_3$ (curve *E*) in a concentration comparable with that in plasma; the curve, however, can be superimposed on that for metaphosphoric acid alone, and it can be concluded that thiosulphate had no appreciable effect. It is of interest to note also that stabilization of the indophenol colour did not occur when ascorbic acid was added to trichloroacetic acid (curve *F*). Similar results were obtained when the indophenol was added to metaphosphoric acid filtrates of plasma. With normal plasma the reaction with the dye was complete in 30 sec. (curve *G*) and this also occurred when the plasma was treated with H_2S which was subsequently removed by N_2 (curve *H*). This curve shows that the H_2S, used in this way, does not interfere with the determination of ascorbic acid, although if the removal of H_2S is incomplete (curve *J*) stabilization of colour is delayed and estimation of ascorbic acid is impossible. The general conclusion is that with spectrophotometer readings taken 30–60 sec. after addition of the dye, with metaphosphoric acid but not trichloroacetic acid as the protein precipitant and with no residual H_2S, the readings represent ascorbic acid concentrations, and that the presence of interfering substances is indicated by a steady change of spectrophotometer readings during the chosen period.

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To test the reproducibility of results, twenty determinations were made on a solution of pure ascorbic acid: the mean result was 1·81 ± 0·025 mg./100 ml., the range being 1·77–1·83; similarly, a sample of plasma with added ascorbic acid gave twenty results between 2·09 and 2·13 mg./100 ml., with a mean of 2·11 and S.D. of 0·019. At a lower ascorbic acid concentration the figures for twenty replicate determinations were: mean, 0·43 mg./100 ml.; S.D., 0·023; range, 0·40–0·48.

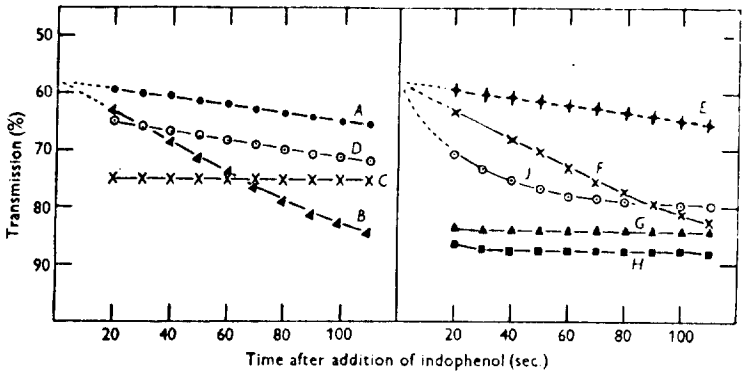

Fig. 1. The effect of time on the transmission at 480 mμ. of 1·0 ml. of the indophenol solution (4 mg./100 ml.) in the presence of 2·0 ml. of various reagents. *A*, metaphosphoric acid 3% (w/v); *B*, trichloroacetic acid 5% (w/v); *C*, HPO_3 3% (w/v) plus ascorbic acid, 1 mg./100 ml.; *D*, HPO_3 3% (w/v) + H_2S; *E*, HPO_3 3% (w/v) + $Na_2S_2O_4$, 8 mg./100 ml.; *F*, trichloroacetic acid 5% (w/v) + ascorbic acid, 1 mg./100 ml.; *G*, 2 ml. plasma + 3 ml. HPO_3, 3% (w/v); *H*, as *G*, after treatment with H_2S and removal of excess H_2S by N_2; *J*, as *H*, but with H_2S incompletely removed.

The results of recovery experiments when pure ascorbic acid was added to plasma are shown in Table 1. Recoveries were always within 0·05 mg. ascorbic acid/100 ml.

Table 1. *Recovery of ascorbic acid added to human plasma using the indophenol method*

Ascorbic acid (mg./100 ml. plasma)			
Added	Found	Calculated	Difference
—	0·15	—	—
0·25	0·37	0·4	−0·03
0·5	0·64	0·65	−0·01
1·0	1·17	1·15	+0·02
1·5	1·70	1·65	+0·05
2·0	2·20	2·15	+0·05

Invariably the results obtained by the method of Roe & Keuther (1943) were higher than those by the method using the indophenol, although both methods were standardized against the same original solution of pure ascorbic acid. A similar difference was found in the plasma from subjects who had been given a diet rich in vitamin C for a few weeks. These results are shown in columns 4 and 5 of Tables 2 and 3. In the normal fasting subjects on an ordinary diet, the mean ratio of plasma ascorbic acid determined by the indophenol method to the 'total ascorbic acid' determined by the method of Roe & Keuther (1943) was 0·68 ± 0·15 (Table 2, column 6). After a period of diet rich in vitamin C, the ratio became 0·82 ± 0·06 (Table 3, column 6), the absolute differences remaining about the same.

There are several possible explanations of this phenomenon other than the obvious one that the difference represents dehydroascorbic acid and or diketogulonic acid existing in the circulating plasma. Oxidation of ascorbic acid to dehydroascorbic acid and subsequent change to diketogulonic acid may occur spontaneously after withdrawal of blood or during the manipulation involved in the indophenol method prior to the estimation. Alternatively, interfering substances may be reacting with 2:4-dinitrophenylhydrazine in the method of Roe & Keuther (1943), giving a falsely high figure for the concentration of total ascorbic acid. Indeed, both possibilities may be involved.

In regard to the first of these suggestions, the stability of ascorbic acid was investigated in blood, plasma and plasma filtrate. For this purpose, venous blood was withdrawn into an oiled syringe; part of the sample was allowed to stand at room temperature with occasional mixing, the remainder being used for the immediate determination of plasma total ascorbic acid and of ascorbic acid by the method of Roe & Keuther (1943), and by the indophenol method respectively. After about 60 and 120 min., further determinations were made by both methods. Similar experiments were carried out on plasma separated immediately after the withdrawal of blood. A third series was done on plasma filtrate prepared as quickly as possible after the withdrawal of the blood. The earliest determination possible in any of these experiments was about 30 min. after withdrawal. Typical results of these experiments are shown in Fig. 2, where it is seen that from the time of the first determination there is a very slow fall in the apparent ascorbic acid con-

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Table 2. *Concentration of vitamin C in the plasma of normal fasting subjects taking an ordinary diet*

Subject no. (1)	Sex (2)	Age (yr.) (3)	Indophenol method. Ascorbic acid (mg./100 ml.) (4)	Method of Roe & Keuther. 'Total ascorbic acid' (mg./100 ml.) (5)	Ratio: Indophenol method / Roe & Keuther method (6)	Ratio: H_2S-indophenol method / Roe & Keuther method (7)
1	M.	28	0·50	0·75	0·67	—
2	M.	24	0·40	0·60	0·67	—
3	M.	30	0·78	0·85	0·91	1·05
4	F.	21	0·30	0·55	0·55	1·04
5	M.	23	0·14	0·40	0·35	0·98
6	F.	28	0·35	0·50	0·70	1·04
7	F.	35	1·32	1·56	0·84	1·00
8	F.	21	0·32	0·50	0·64	1·00
9	F.	21	1·0	1·30	0·77	1·00
10	F.	23	0·50	0·70	0·71	—
Mean	—	—	—	—	**0·68**	**1·01**
S.D.	—	—	—	—	±0·15	±0·03

Table 3. *Concentration of vitamin C in the plasma of normal fasting subjects taking a diet rich in vitamin C*

Subject no. (1)	Sex (2)	Age (yr.) (3)	Indophenol method. Ascorbic acid (mg./100 ml.) (4)	Method of Roe & Keuther. 'Total ascorbic acid' (mg./100 ml.) (5)	Ratio: Indophenol method / Roe & Keuther method (6)	Ratio: H_2S-indophenol method / Roe & Keuther method (7)
1	F.	28	0·84	1·05	0·76	1·00
2	M.	23	0·44	0·60	0·73	0·96
3	M.	30	1·07	1·25	0·86	1·00
4	F.	25	0·94	1·10	0·85	1·06
5	F.	20	1·56	1·90	0·81	1·05
6	F.	21	0·94	1·10	0·85	1·00
7	M.	24	1·97	2·22	0·88	0·99
Mean	—	—	—	—	**0·82**	**1·01**
S.D.	—	—	—	—	±0·06	±0·03

centration in blood, blood plasma, and plasma filtrate under these conditions. The rate of loss of ascorbic acid is slightly greater when the plasma is left in contact with red cells, than when it has been separated. The total ascorbic acid does not alter during the period of these experiments or, indeed, for several hours afterwards.

If the decay curve before the time of the first determination is the same as it is afterwards, extrapolation to zero time should provide an estimate of ascorbic acid at that time. It is evident from Fig. 2 that the plasma concentrations of ascorbic acid at zero time obtained by such extrapolation are very little different from the concentrations 30 min. later and are still lower than those of total ascorbic acid by the method of Roe & Keuther (1943). The validity of the assumption that the rate of loss of ascorbic acid between the withdrawal time ($t = 0$) and the earliest determination ($t = 30$ min.) follows the same simple decay curve as it does subsequently is established by the recovery of ascorbic acid and dehydroascorbic acid added to blood plasma or plasma filtrate. Table 1 shows that such recoveries are complete. It is concluded from these experiments that the differences between the plasma concentrations of total ascorbic acid as determined by the method of Roe & Keuther (1943) and those determined by the indophenol method are not due to the oxidation of ascorbic acid following the withdrawal of blood and during the indophenol procedure.

In regard to the second possibility, Roe & Keuther (1943) have satisfactorily excluded many potential interfering substances, either because the colour with 2:4-dinitrophenylhydrazine does not develop under the strongly acid conditions of the estimation, or because they (e.g. glucose) are not present in plasma in sufficient concentrations to interfere significantly with the results.

The nature of the substance or substances responsible for the difference obtained by the two methods was investigated by means of hydrogen sulphide.

Pure H_2S, prepared from Sb_2S_3, was passed for 10 min. through samples of filtrate prepared with metaphosphoric acid from plasma in which determinations by the two methods had already been carried out (i.e. subjects shown in Tables 2 and 3). Excess of H_2S was then removed by blowing

pure N_2 saturated with water vapour through the solution for 2–3 hr., by which time the nitroprusside and lead acetate reactions were negative.

The indophenol method was again applied (the stability of the colour showing absence of interference by hydrogen sulphide), and the ratios of these determinations to the original determinations by the method of Roe & Keuther (1943) are given in column 7 of Tables 2 and 3. In every case, the indophenol-reducing substance has increased to the value given by the Roe & Keuther (1943) method for total ascorbic acid, so that the mean ratio rises from 0·68 or 0·82 to 1·0 ± 0·03 irrespective of the original value. Control experiments showed that when a trichloroacetic acid filtrate of plasma, as used for the Roe & Keuther (1943) method, was treated with hydrogen sulphide and nitrogen prior to oxidation with charcoal, the result did not differ from that obtained by the usual procedure.

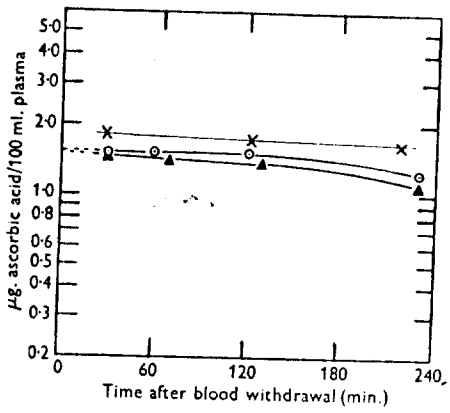

Fig. 2. The effect of time on the apparent ascorbic acid content of plasma. ⊙—⊙, plasma separated immediately after blood withdrawal, samples used for ascorbic acid determination by the indophenol method. (Plasma separated and immediately treated with metaphosphoric acid gave results indistinguishable from these.) ▲--▲, plasma allowed to stand in contact with red cells until required (indophenol method). ×—×, plasma separated immediately after blood withdrawal; 'total ascorbic acid' determined by Roe & Keuther method.

Thus the substance in plasma filtrate, which is responsible for the difference between the ascorbic acid content measured by the indophenol method and the 'total ascorbic acid' measured by the 2:4-dinitrophenylhydrazine method of Roe & Keuther (1943), (*a*) reacts with dinitrophenylhydrazine but not with the indophenol, (*b*) is reducible by hydrogen sulphide to a substance which reacts with the indophenol as rapidly as does ascorbic acid, and (*c*) when calculated as ascorbic acid, accounts precisely for the observed difference. Taking into account the specificities of the two reactions used in the analyses, it is difficult to imagine such behaviour being due to anything except dehydroascorbic acid. Diketogulonic acid also reacts with dinitrophenylhydrazine under the conditions of Roe & Keuther (1943), but may be ruled out since it is not reduced by hydrogen sulphide in acid solution. It may be concluded, with a high degree of probability, that the observed differences are due to the presence of dehydroascorbic acid in the filtrates prepared from plasma by addition of metaphosphoric acid. The extension of this conclusion to native plasma rests partly on the time relationships discussed earlier and partly on the well established fact that in weakly acid solution at room temperature equilibrium between dehydroascorbic acid and diketogulonic acid (with the latter forming about 80% of the equilibrium mixture) is reached only in about 70 hr. Hence if diketogulonic acid were originally present in the plasma it should still be detectable in the acidified plasma filtrate 30 min. later (Herbert, Hirst, Percival, Reynolds & Smith, 1933). The distinction may be of some importance, since at the slightly alkaline reaction of plasma some conversion of dehydroascorbic acid to diketogulonic acid might be expected. The results of the experiments just described suggest, however, that plasma itself contains dehydroascorbic acid but no appreciable amount of diketogulonic acid.

Changes in the plasma concentration of ascorbic acid and dehydroascorbic acid following intramuscular administration of adrenocorticotropic hormone (ACTH)

The known relationship of vitamin C to the adrenal cortex suggested that an investigation of the ratio of dehydroascorbic acid to total ascorbic acid in plasma following administration of ACTH and compound E would be of interest. Further, a consistent alteration in the ratio brought about by these means would strengthen the conclusion that dehydroascorbic acid exists in plasma.

ACTH ('Acthar', Armour Laboratories, Armour and Co., Chicago) was given by intramuscular injection in doses of 100 mg. to four normal fasting and resting subjects. Prior to the injection one or two blood samples were withdrawn. Following the injection, samples of blood were withdrawn at hourly intervals for 4–7 hr., and during this period the subjects were allowed to drink weak tea or coffee which was shown to have no effect upon the concentration of ascorbic acid or dehydroascorbic acid. The same subjects were used as controls about 1 week later, at which time samples were taken at hourly intervals for 4–6 hr. for analysis without hormone administration but otherwise under the same conditions. The activity of the ACTH used was confirmed in all cases by eosinopenic responses of at least 50% within 2–5 hr. after the injection. In addition, H_2S was passed through a sample of the metaphosphoric acid filtrate for 10 min., after which excess H_2S was removed by N_2 and a further estimation of the indophenol-reducing power was made.

Table 4. *Changes in the concentration of plasma ascorbic acid and dehydroascorbic acid following 100 mg. ACTH given by intramuscular injection*

Subject no.	Plasma ascorbic acid (Δ mg./100 ml.)	Plasma ascorbic acid (Δ %)	Plasma dehydroascorbic acid (Δ mg./100 ml.)	Plasma dehydroascorbic acid (Δ %)	Plasma 'total ascorbic acid' (Δ mg./100 ml.)	Plasma 'total ascorbic acid' (Δ %)
1 B.S.	+0·42	+42	−0·17	−56	+0·25	+19
2 J.R.	+0·25	+37	−0·13	−65	+0·15	+13
3 M.K.	+0·25	+76	−0·18	−100	+0·05	+10
4 E.B.	+0·23	+13	0*	0	+0·25	+14
Mean	—	+42	—	−74	—	+14

* No fasting dehydroascorbic acid present.

Table 5. *The effect of treatment with hydrogen sulphide on the ratio of ascorbic acid (indophenol method) to 'total ascorbic acid' (Roe & Keuther method) after administration of ACTH*

Treatment of subject	Ratio: Indophenol method / Roe & Keuther method	Ratio: H_2S-indophenol method / Roe & Keuther method	Ratio: Indophenol method / Roe & Keuther method	Ratio: H_2S-indophenol method / Roe & Keuther method
Fasting	0·76	1·0	0·65	1·0
ACTH 1 hr. previously	0·86	0·98	1·0	1·0
ACTH 2 hr. previously	0·86	1·01	1·01	1·01
ACTH 3 hr. previously	0·87	0·95	0·95	0·96
ACTH 4 hr. previously	0·92	0·96	0·85	0·97
ACTH 5 hr. previously	0·98	0·99	—	—
Mean	—	0·98	—	0·99

The results are summarized in Table 4 and one case is shown graphically in Fig. 3. Following the administration of ACTH, a rise in total ascorbic acid occurred in all four subjects as determined by the method of Roe & Keuther (1943). The rise started within 1 hr. of the injection and reached a maximum in 2–4 hr., corresponding approximately in time to the maximum eosinopenia. The mean percentage change in total ascorbic acid concentration (ascorbic acid + dehydroascorbic acid) was +14, absolute elevations varying from 0·05 to 0·25 mg./100 ml. plasma. Ascorbic acid itself, as determined by indophenol reduction, also showed increases of from 13 to 76 % of the fasting level, and these increases were maximal at the time corresponding to the peak of total ascorbic acid concentration. The absolute increments in ascorbic acid concentration varied from 0·25 to 0·41 mg./100 ml. plasma. The larger increments in ascorbic acid as compared with total ascorbic acid (ascorbic acid + dehydroascorbic acid) resulted in a diminution in the absolute concentration of dehydroascorbic acid amounting to 0·13–0·18 mg./100 ml. in three subjects (56–100 %). In the fourth subject no fasting dehydroascorbic acid existed prior to the injection and none appeared following it. The control experiments in which no ACTH was given showed only very slight irregular alterations in the plasma concentration of total and reduced ascorbic acid. The results obtained following the passage of hydrogen sulphide through the metaphosphoric acid plasma filtrate are shown in Table 5, which gives the ratios of ascorbic acid to total vitamin C before and after injection of ACTH for subjects B. S. and M. K. The disappearance of dehydroascorbic acid in these subjects is shown by the rise in the ratio from 0·76 to 0·98 and from 0·65 to 1·0 respectively; the reappearance of dehydroascorbic acid in M. K. is evidenced by the gradual fall of the ratio to 0·85, 4 hr. after the injection. Hydrogen sulphide had the effect of making the ratio very nearly 1·0 whatever its value in the original plasma. It is important to note that when the original ratio is 1·0, hydrogen sulphide does not increase it.

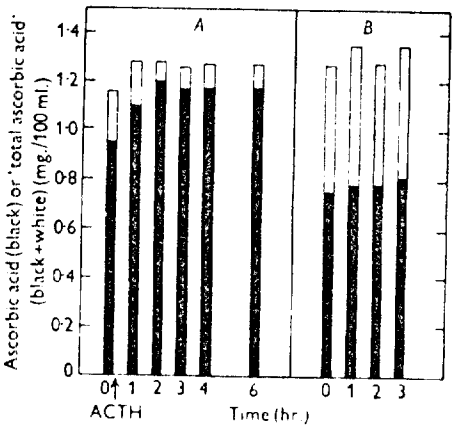

Fig. 3. The ascorbic acid (black columns) and dehydroascorbic acid (white columns) of human plasma. *A*, after intramuscular injection of 100 mg. ACTH (time measured from the time of ACTH injection), and *B*, in successive hours without ACTH administration.

Changes in the plasma concentration of ascorbic acid and dehydroascorbic acid following oral administration of cortisone acetate

The changes in plasma ascorbic acid and dehydroascorbic acid following administration of ACTH are not easily interpreted. ACTH is known to diminish the concentration of ascorbic acid in the adrenal gland (e.g. Sayers, Sayers, Liang & Long, 1946). This might explain the increase in the plasma total ascorbic acid, but does not by itself account for the decrease in dehydroascorbic acid. In addition, however, the increased secretion of adrenocortical hormones, acting peripherally, might produce effects upon vitamin C metabolism capable of accounting for the latter phenomenon. In order to dissociate such central from peripheral effects, the changes in blood concentration following the oral administration of 350 mg. of cortisone acetate were investigated in five normal subjects, one of whom (J.R.) had already been examined following ACTH administration.

As before, the subjects were fasting and at rest, though weak tea or coffee was allowed. One or two samples of blood were withdrawn prior to giving the cortisone, after which samples were taken into oiled syringes at 30 min., 1 hr., and then at hourly intervals for 4–6 hr. The chemical determinations were made as before, and eosinophils were counted.

The results are shown graphically for two subjects in Fig. 4 and in Table 6 they are summarized for the five subjects in terms of the maximum changes occurring in the plasma concentration of ascorbic and dehydroascorbic acids. These occurred between 0·5 and 2 hr. after administration of cortisone and corresponded in time to the maximal eosinopenia (i.e. 50%). The mean increase in plasma ascorbic acid was 38%, corresponding to an absolute rise in concentration ranging from 0·1 to 0·34 mg./100 ml. of plasma. There was no rise in the concentration of total vitamin C in any of the subjects and the maximum rise in concentration of ascorbic acid exactly equalled the original concentration of dehydroascorbic acid, so that all the dehydroascorbic acid in the plasma temporarily disappeared. The ratio of ascorbic acid to total vitamin C for subjects J.R. and M.McI. at various times following administration of the cortisone is given in Table 7,

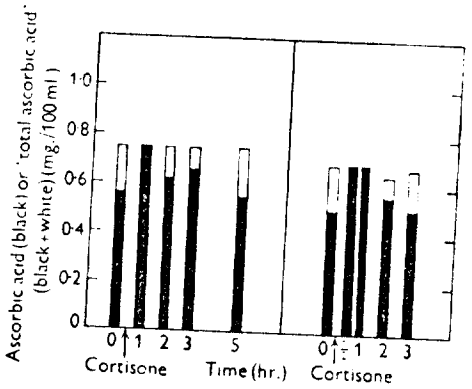

Fig. 4. The effect on the ascorbic acid (black columns) and dehydroascorbic acid (white columns) of human plasma of oral administration of 350 mg. of cortisone acetate given immediately after withdrawal of the control blood sample. Two cases.

Table 6. *Changes in concentration of plasma ascorbic acid and dehydroascorbic acid following 350 mg. cortisone given orally*

Subject no.	Plasma ascorbic acid (Δ mg./100 ml.)	Plasma ascorbic acid (Δ %)	Plasma dehydroascorbic acid (Δ mg./100 ml.)	Plasma dehydroascorbic acid (Δ %)	Plasma 'total ascorbic acid' (Δ mg./100 ml.)	Plasma 'total ascorbic acid' (Δ %)
1 H.L.	+0·34	+44·0	—	—	—	—
2 J.R.	+0·20	+36·0	−0·19	95	0	0
3 M.McI.	+0·10	+22·0	0·10	100	0	0
4 H.A.	+0·17	+33·0	0·17	100	0	0
5 R.C	+0·10	+55·0	0·10	100	0	0
Mean	—	+38·0		99		0

Table 7. *The effect of treatment with hydrogen sulphide on the ratio of ascorbic acid (indophenol method) to 'total ascorbic acid' (Roe & Keuther method) after administration of 350 mg. cortisone given orally*

Treatment of subject	Subject J.R., 30 yr., M. Ratio: Indophenol method / Roe & Keuther method	Subject J.R., 30 yr., M. Ratio: H_2S Indophenol method / Roe & Keuther method	Subject M.McI., 45 yr., F. Ratio: Indophenol method / Roe & Keuther method	Subject M.McI., 45 yr., F. Ratio: H_2S-Indophenol method / Roe & Keuther method
Fasting	0·74	1·00	0·82	1·02
Cortisone, 1 hr. previously	1·00	0·99	1·00	0·98
Cortisone, 2 hr. previously	0·83	1·0	0·95	
Cortisone, 3 hr. previously	0·87	1·01	0·94	1·04
Cortisone, 4 hr. previously	0·74	0·98	0·92	1·02
Mean	—	1·0		1·02

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which also shows the effect of hydrogen sulphide upon this ratio. Without hydrogen sulphide treatment the ratio rises to 1·0 about 1 hr. after administration of cortisone (i.e. dehydroascorbic acid has disappeared) and subsequently falls as plasma dehydroascorbic acid reappears. Treatment with hydrogen sulphide gives a ratio approximately 1·0 (but never significantly greater), irrespective of the original value, so that the changes are legitimately interpreted as indicating disappearance of dehydroascorbic acid.

DISCUSSION

The possibility of the existence of significant concentrations of dehydroascorbic acid in blood or tissues has received little attention in the past. The bare statement that both dehydroascorbic acid and diketogulonic acid occur in plasma and urine was made by Chen & Sebaiek (1950). Nyden (1948) studied alterations in ascorbic and dehydroascorbic acids in rat tissues and plasma following their injection with *Trypanosoma hippicum*. She used the method of Roe & Oesterling (1944), devised originally for the estimation of dehydroascorbic acid in plants, to estimate tissue and plasma dehydroascorbic acid concentrations. In our hands this method proved unsuitable for the determination of plasma dehydroascorbic acid, since reduction of dehydroascorbic acid occurred in the presence of all samples of thiourea (1 %, w/v) available to us, and oxidation occurred when thiourea was omitted. The concentration of thiourea required to maintain the naturally occurring ratio between the reduced and oxidized forms of the vitamin was unpredictable. The almost complete absence in the literature of a simple comparative study of the method of Roe & Keuther (1943) and one estimating the vitamin in the reduced form (e.g. by indophenol or methylene blue) is surprising. Lowry, Lopez & Bessey (1945) compared the micro-indophenol method of Mindlin & Butler (1937–8) with their own micro-adaptation of the method of Roe & Keuther (1943), using 0·01 ml. of serum but without making any direct comparison with the micromethod of Roe & Keuther (1943). They found no significant difference between the results of the two techniques when applied to serum, but it is questionable whether their micromodification was sensitive enough to detect the small differences reported in this paper. In addition, it has been established by Friedman, Rumin & Kees (1938), Farmer & Abt (1938) and by Cushman & Butler (1938) that, as we also have found, potassium cyanide employed in the method of Mindlin & Butler (1937–8) invalidates the results; this compound was presumably used by Lowry *et al.* (1945). As long ago as 1935, Martini, Bonsignore & Pinotti claimed that dehydroascorbic acid existed in significant concentration in guinea pig tissues, but the use of trichloroacetic acid in tissue extraction may invalidate these results, at least partly, since trichloroacetic acid itself appears to decolorize indophenol.

The source of the increment in total plasma vitamin C following intramuscular ACTH is unknown. Vogt (1948) was unable to detect significantly increased amounts of vitamin C in venous blood leaving the adrenal cortex of dogs when the gland was directly stimulated. It can be said, however, that the amount of ascorbic acid reported to disappear from the adrenal cortex after ACTH administration is sufficient, if added to the extracellular body fluids, to increase the ascorbic acid concentration by the amounts observed.

The temporary diminution or disappearance of plasma dehydroascorbic acid is an effect common both to intramuscular ACTH and oral cortisone. The fact that total plasma vitamin C concentration does not alter significantly following cortisone administration indicates that one of the essential responses to this adrenocortical hormone is an activation of the mechanism for the reduction of dehydroascorbic acid.

Simple *in vitro* experiments in which cortisone and ACTH are incubated with dehydroascorbic acid either in solution or in plasma were carried out in the course of this work but have not been reported in detail in the experimental part. They showed clearly that the observed apparent reduction of dehydroascorbic acid is not a direct chemical effect of the circulating hormone. Borsook *et al.* (1937) have shown that normal human plasma does not possess the power of reducing dehydroascorbic acid. We have confirmed this and shown further that the power of such reduction is not acquired by plasma following ACTH or cortisone administration. The activation is, therefore, likely to occur within the cells either of the blood or of tissues and it is possible that the mechanism involves glutathione. Experiments to test this possibility are now in progress.

SUMMARY

1. A comparison of the method of Roe & Keuther (1943) with a procedure using 2:6-dichlorophenol-indophenol for determination of 'total ascorbic acid' and ascorbic acid respectively provides results which suggest that a significant part of the vitamin C in human plasma is present as dehydroascorbic acid.

2. This possibility is further substantiated by the fact that the differences obtained in the concentrations of plasma vitamin C by the two methods are completely removed by treating plasma filtrate with hydrogen sulphide prior to using the indophenol method, irrespective of the magnitude of the difference which existed before such treatment.

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3. Taking into account the specificities of the two methods and the reactions they involve, it is difficult to explain such results except as being due to the presence of dehydroascorbic acid. These procedures do not suggest the presence of diketogulonic acid in human plasma.

4. The administration of adrenocorticotropic hormone to human subjects resulted in a rise in the concentration of plasma total ascorbic acid and of plasma ascorbic acid with a diminution in the concentration of plasma dehydroascorbic acid 2–4 hr. after intramuscular injection.

5. The oral administration of cortisone to human subjects resulted in no rise in the concentration of plasma total ascorbic acid, but in a rise in ascorbic acid of such a magnitude that dehydroascorbic acid temporarily disappeared from the plasma 0·5–2 hr. after administration.

6. The significance of these alterations is discussed both with respect to the origin of the increment of total ascorbic acid appearing in the plasma and to the mechanisms involved in the reduction of dehydroascorbic acid to ascorbic acid.

We wish to thank the Medical Research Council for a grant which helped to defray the cost of materials and technical assistance as well as for a supply of hormones.

Note added in proof. In a few experiments it has now been found that, following the administration of cortisone or ACTH and coincident with the resulting diminution of the plasma dehydroascorbic acid concentration, there is a decrease in the reduced glutathione concentration of the whole blood. When, later, the original dehydroascorbic acid concentration is restored, the reduced glutathione of the blood also increases. The sum of the reduced and oxidized glutathione remains constant throughout.

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Influence of Sex and Dietary Ascorbic Acid on Nitrite-Induced[1] Methemoglobinemia in Japanese Quail (34646)

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Increased attention is being given to the potential ecological hazards associated with high nitrate concentrations in food sources and water supplies. The toxicity of nitrates in animals and man is primarily due to its reduction to nitrite prior to, or during, ingestion with the accompanying conversion of hemoglobin into methemoglobin (1). Methemoglobinemia is the condition in which the ferrous ion of hemoglobin is oxidized to ferric ion, thereby impairing the capacity of the blood to transport oxygen (1).

Sell *et al.* (2) observed reduced feed consumption and increased methemoglobin concentrations of the blood within 8 hr after feeding male chicks diets containing 0.4% nitrite.

The study reported here was designed to determine the effects of dietary nitrite and ascorbic acid upon the formation of methemoglobin in adult male and female Japanese quail (*Coturnix coturnix* Japonica) and upon nitrite deposition in the egg. Injection, or in some instances, oral administration of vitamin C has been shown to reduce methemoglobin levels (3). The Japanese quail was chosen as the experimental animal for use in these studies because of their limited space requirements and their relatively rapid maturation.

Experimental Methods. Japanese quail were obtained from the Department of Poultry Science, Cornell University and were placed in a commercial brood unit at one day of age. They were divided into 4 groups with 13 quail chicks in each group and were fed a purified diet consisting of: glucose, 53.89; isolated soybean protein, 30.00; corn oil, 5.00; cellulose, 3.00; DL-methionine, 0.75; glycine, 0.30; and required vitamins and minerals, 7.06 (4). At 3 weeks of age 0.5% L-ascorbic acid was added at the expense of glucose in the diets of two of the groups. Since female Japanese quail begin to lay eggs at about 5 weeks of age, the dietary calcium was then increased to 2.4% substituted for glucose in all groups. At 15 weeks of age, one group of quail fed the ascorbic acid and one group fed the basal diet without the additional dietary ascorbic acid were given 0.5% dietary nitrite as $NaNO_2$ for 1 week.

Methemoglobin and hemoglobin concentrations of blood obtained by heart puncture from 4 to 160 hr after the nitrite regimen commenced were determined by the modified (5) method of Evelyn and Malloy (6). Egg nitrite deposition was determined by the method developed by Diven *et al.* (7).

Results. When nitrite was fed for 1 week to the 15-week-old Japanese quail, body weights of both males and females were depressed (Fig. 1). Food intake was concomi-

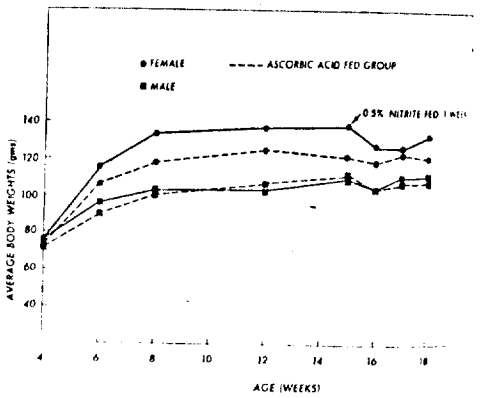

FIG. 1. Growth curves of male and female Japanese quail fed ascorbic acid and nitrite.

[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1741.

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TABLE I. Hemoglobin and Methemoglobin Concentration of Male and Female Japanese Quail as Influenced by Dietary Ascorbic Acid and Nitrite.

Sex	Ascorbic[a] acid fed	Nitrite[a] fed	Hemoglobin[b] (g/100 ml)	Methemoglobin[b] (g/100 ml)	Methemoglobin[b] (% of hemoglobin)
Male	0	0	13.28 ± 0.89[c]	0.51 ± 0.09	4.15 ± 0.27 (5)[d]
	0	+	11.28 ± 0.81	2.09 ± 0.38	15.16 ± 2.99 (6)
	+	0	12.23 ± 0.30	0.52 ± 0.03	4.27 ± 0.26 (5)
	+	+	12.13 ± 0.94	1.60 ± 0.41	13.17 ± 2.89 (7)
Female	0	0	10.75 ± 1.48	1.03 ± 0.03	10.07 ± 0.79 (3)
	0	+	10.26 ± 0.49	2.68 ± 0.80	26.54 ± 7.86 (6)
	+	0	10.36 ± 0.18	1.54 ± 0.20	14.80 ± 1.81 (5)
	+	+	9.90 ± 0.41	1.89 ± 0.26	19.33 ± 2.79 (5)

[a] Ascorbic acid added to diets (+) at 3 weeks of age. Nitrite fed for 7 days at 15 weeks of age.

[b] Values obtained during week of nitrite regimen.

[c] Mean ± SE.

[d] Number of birds determined in each group.

tantly depressed from a daily intake of 16–17 g/birds to 11–12 g. Quail not fed the nitrite regimen maintained their weight with an average of 2–5-g increase during this period.

The blood hemoglobin and methemoglobin values of male and female quail fed ascorbic acid and nitrite, and those of the controls, are presented in Table I. Hemoglobin values for females were lower and the methemoglobin blood levels were higher than those in the male quail. Analysis of variance of the data show that the methemoglobin values of either control or nitrite fed females were significantly ($p<0.05$) increased, as percentage of the total hemoglobin content, compared to the male quail fed the same diet. Without nitrite feeding, female quail exhibited a twofold increase of blood methemoglobin compared to males. When nitrite was added to the diet, a significant increased ($p<0.05$) level of blood methemoglobin occurred in all of the animals. This level remained relatively constant from 4 to 160 hr after initiation of nitrite intake. Dietary ascorbic acid had no significant ($p>0.05$) effect on blood methemoglobin in either sex regardless of nitrite intake.

Nitrite first appeared in the quail eggs about 10 hr after nitrite feeding started and continued at reduced levels up to 140 hr after nitrite intake ceased. Nitrites (as μg nitrite-nitrogen/g of egg) in control eggs averaged 0.29 ± 0.02 as compared to 0.33 ± 0.06 in eggs produced from quail hens fed ascorbic acid. Ascorbic acid has no significant effect on nitrite deposition in eggs of nitrite fed quail.

Discussion. Methemoglobin is normally present in the blood of animals and man (1). Von Issekutz (8) observed that cats normally have a methemoglobin concentration ranging between 4 and 17% of the total hemoglobin level in the blood. These levels are quite similar to the range of methemoglobin values of the Japanese quail in this study, dependent on the sex, not treated with nitrite. Sex hormones have a marked effect on methemoglobinemia, since there was approximately a twofold increase in methemoglobin blood levels of the female quail as compared with the similarly treated males. The observation of Tanaka and Rosenberg (9) that androgens increase hemoglobin blood levels in chickens, offers a possible explanation for the higher blood hemoglobin levels in the male Japanese quail than in the females.

In agreement with Sell *et al.* (2), results using male chicks, nitrite intake reduced body weights and food consumption concurrently with the development of a methemoglobinemia in male and female Japanese quail. The methemoglobinemia together with nitrite deposition into eggs, were not affected by dietary ascorbic acid, although this vitamin has been used therapeutically for congen-

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ital methemoglobinemia (3). These results are in agreement with Kilgore *et al.* (10) who reported that guinea pigs fed diets low in ascorbic acid did not show increased susceptibility to nitrite toxicosis.

Summary. Japanese quail fed diets containing 0.5% nitrite developed a methemoglobinemia together with reduced food consumption and depressed body weights. Nitrite also was deposited in eggs, and continued to be present for about 140 hr after nitrite feeding stopped. Female Japanese quail, either controls or nitrite treated, were observed to have less blood hemoglobin, with approximately a twofold increase in blood methemoglobin than males from the equivalent treatments. Ascorbic acid added at 0.5% to the diet had no effect on methemoglobinemia or egg nitrite deposition.

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Nitrite-induced Methemoglobinemia in Guinea Pigs: Influence of Diets Containing Beets with Varying Amounts of Nitrate, and the Effect of Ascorbic Acid, and Methionine[1,2]

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ABSTRACT Experiments were conducted with young, male guinea pigs to investigate the influence of feeding a vegetable with naturally occurring low and high amounts of nitrate, and the influence of dietary supplementation of ascorbic acid and methionine on nitrite-induced methemoglobinemia. Freeze-dried red table beets were incorporated into experimental diets. They contained either a low or high amount of nitrate from manipulation of nitrogen fertilization rates during their growing period. High nitrate-containing beet diets, or nitrate added to diets, did not induce methemoglobinemia. High nitrate diets fed to guinea pigs enhanced nitrite-induced methemoglobinemia. Low nitrate-containing beet diets seemed to "protect" guinea pigs from nitrite toxicosis. Purified diets containing 1% added L-ascorbic acid slightly reduced nitrite-induced methemoglobin blood levels of normal guinea pigs compared to those fed 0.02% ascorbic acid, but there was an additional 50% reduction when 1% methionine and 1% or 2% ascorbic acid were included in the diet. J. Nutr. *103:* 419–424, 1973.

INDEXING KEY WORDS methemoglobinemia · beets · nitrate · nitrite · ascorbic acid · methionine

Nitrite ingestion from foods or water supplies constitutes a hazard of potential toxicity (1). Nitrites and nitrates have been shown to react with various amines or amides to form carcinogenic nitroso compounds in experimental animals (2, 3); however, the acute symptom of nitrite toxicity, i.e., oxidation of hemoglobin to methemoglobin, has occurred in livestock (4) and humans, especially infants under 4 months of age (5, 6).

It is generally assumed that unless nitrates are reduced to nitrites there is little concern regarding methemoglobin development. Indeed, most cases of infant methemoglobinemia have been associated with well water whereby nitrites were present in the drinking water, or were formed from nitrates in vivo (5). Although high amounts of nitrates can occur in certain vegetables as beets and spinach grown under excessive levels of nitrogen fertilization (7, 8), only a relatively few cases of infant methemoglobinemia have been observed with intake of high nitrate spinach (6, 9, 10). Natural compounds in plant foods could be, in part, protective against methemoglobin formation. Dietary ascorbic acid has been associated with reduced methemoglobin development in guinea pigs (11), but other evidence (12, 13) does not corroborate these data. Methionine may also have an effect on methemoglobin development (14).

The present study was initiated to investigate a) the influence of feeding table beets, containing low or high naturally occurring nitrates on methemoglobinemia, and b) the influence of dietary ascorbic

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[2] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1759.

TABLE 1

Nitrate and nitrite content in beet and purified diets

Diets[1]	Nitrate-nitrogen	Nitrite-nitrogen
	ppm	*ppm*
High nitrate beet	1,998	2.5
Low nitrate beet	242	1.7
High nitrate purified	1,798	3.0
Low nitrate purified	148	4.0

[1] Details of complete formulation are in text footnote 4.

acid and methionine on nitrite-induced methemoglobinemia in young male guinea pigs.

MATERIALS AND METHODS

Young male albino guinea pigs, 180 to 200 g, of the Hartley strain [3] were caged in pairs in suspended stainless steel wire screen units. Distilled water was supplied ad libitum. The guinea pigs were fed the experimental diets [4] for 9 hours each day. The red table beets used in experiment 1, (*Beta vulgaris L.*) cv. "Ruby Queen," were grown in experimental plots, and nitrogen was either not applied or applied as NH_4NO_3 at 450 kg of nitrogen per hectare. The beets were washed, peeled, diced, freeze-dried, and then finely chopped for incorporation into the experimental diet.

Nitrate-nitrogen determinations of the beets were carried out potentiometrically (8), and nitrite nitrogen was determined by spectrophotometric method of Kamm et al. (16). Ascorbic acid content of the beets was determined by the indophenol-xylene extraction method of Robinson and Stotz (17). Methionine content of the beets was determined by a modified gas chromatographic method (18).

Blood was obtained via cardiac puncture from the guinea pigs under light ether anesthesia, after feeding the diets for 2.5 to 4 weeks. Nitrite dosing was given with a number 20 dosing needle [5] 2.5 hours after daily feeding started, either per os or stomach intubated. Blood hemoglobin and methemoglobin was determined by the procedure of Evelyn and Malloy (19). Blood plasma nitrite was measured spectrophotometrically by the method described by Diven et al. (20). Blood ascorbic acid was determined as described by Lench and Lewis (21).

Statistical comparisons of treatment means in the tables were computed according to Duncan (22).

RESULTS

Experiment 1. The amount of nitrate present in the beet diets, occurring from high fertilization of the growing table beets, was added as sodium nitrate to the purified diets. The analyses for both nitrate and nitrite of the four diets are presented in table 1. After adjusting to both diet and meal feeding schedule, the guinea pigs gained, on the average, 1.5 to 2.0 g per day fed the beet diets, and 2.3 to 2.9 g per day fed either purified diet. Blood samples from all the guinea pigs in all treatment groups were analyzed for methemoglobin. There were no differences in blood methemoglobin level of the animals fed the four diets. The levels ranged from 1.0 ± 0.5 to 1.8 ± 0.7 (as % of total hemoglobin).

As observed in table 2, when the guinea pigs were orally dosed with the low level of sodium nitrite (7.6 mg nitrite-nitrogen per kg body weight), the blood methemoglobin levels were significantly ($P < 0.05$) lowered in the animals fed the low nitrate beet or purified diets compared to the respective high nitrate diets. When the nitrite dose was doubled, blood methemoglobin levels were significantly lowered ($P < 0.05$) only in the guinea pigs fed the low nitrate beet diet. When the level of nitrite intake was tripled, i.e., 22.8 mg/kg, the relatively high blood methemoglobin levels of guinea pigs fed all diets were the same (table 3). Death occurred within each dietary group at this high nitrite dose, but there was only 20% mortality in the group of guinea pigs fed the low nitrate beet diet compared to

[3] Thorp Industries, Inc., White Bear Lake, Minn.

[4] All diets were, in general, formulated according to Reid and Briggs (15). Details of the formulations: Experiment 1. Freeze-dried beets added at 40% of diet, substituted by weight for carbohydrates and 2% of the cellophane in the purified diet. No ascorbic acid added to the beet diet. The purified diet contained (in %): casein, 28.0; cellophane spangles, 15.0; dextrin, 20.0; sucrose, 10.3; glucose, 7.8; corn oil, 7.5; choline chloride (50%), 0.4; potassium acetate, 2.5; magnesium oxide, 0.5; L-arginine·HCl, 1.0; salt mixture (15), 6.0; vitamin mixture (15), 1.0; ascorbic acid, 0.04. Experiment 2. Purified diets were identical with experiment 1 except that isolated soybean protein was substituted for casein and ascorbic acid was not added to the basal diet. Ascorbic acid and DL-methionine were added as separate dietary treatments, substituted by weight for the carbohydrates, as indicated in tables 4 and 5.

[5] Popper and Sons, Inc., New York.

TABLE 2

Nitrite-induced methemoglobinemia of guinea pigs fed high and low nitrate diets

Diets	Nitrite dose[1]	Hemoglobin[2]	Methemoglobin[2]
	mg/kg body wt	*g/100 ml*	*% of hemoglobin*
High nitrate beet	7.6	10.4±0.6[3]	20.3±3.1[ab4]
	15.0	10.4±0.5	43.9±4.3[y]
Low nitrate beet	7.6	11.4±0.4	12.0±1.7[c]
	15.0	11.2±0.2	27.1±3.6[x]
High nitrate purified	7.6	8.6±0.8	21.1±2.9[a]
	15.0	10.6±0.2	44.9±2.8[y]
Low nitrate purified	7.6	11.6±0.2	14.0±1.4[bc]
	15.0	10.5±0.4	46.0±3.3[y]

[1] Expressed as nitrite-nitrogen; 8 to 10 guinea pigs per group. [2] 1.5 hours post dose. [3] Averages ±SEM. [4] Letter superscripts that are the same, within each nitrite dose level, indicate nonsignificance ($P > 0.05$).

44 to 60% mortality in the three other dietary groups. The levels of blood nitrite nitrogen, dependent on nitrite dose level, were similar in all dietary groups.

Experiment 2. After feeding the isolated soybean protein diets without ascorbic acid for 2.5 weeks, the guinea pigs started showing the initial symptoms of deficiency (23). The mean hemoglobin and methemoglobin blood values of the nitrite-dosed guinea pigs fed diets containing either 0, 0.02, or 1% L-ascorbic acid with 0 or 1% DL-methionine are presented in table 4. Slight anemia was observed in the ascorbic acid-deficient animals. Only when the guinea pigs received the highest amount of dietary ascorbic acid, together with additional methionine, was the nitrite-induced methemoglobinemia significantly ($P < 0.05$) reduced compared to that in guinea pigs receiving no ascorbic acid or supplemental methionine. This did not occur when the diet contained 1% ascorbic acid without supplemental methionine. In addition there was observed decreased methemoglobinemia in the guinea pigs fed supplemental methionine within each of the 0.02% and 1% ascorbic acid-treated groups.

The data presented in table 5 are the blood hemoglobin, methemoglobin, ascorbic acid, and body weights of another group of guinea pigs fed 0, 1%, and 2% ascorbic acid with the same dietary level of methionine. The nitrite was administered per os, and blood was analyzed 1 hour post dose. The 1% additional dietary ascorbic acid and methionine again significantly ($P < 0.05$) reduced blood methemoglobinemia. Adding 2% ascorbic acid and methionine to the diets did not appear to further reduce blood methemoglobin levels. Blood ascorbic acid was correlated with dietary ascorbic acid and the scorbutic guinea pigs exhibited lowered body weights.

DISCUSSION

Our results indicate that guinea pigs fed high nitrate-containing beets, or diets containing added nitrates, enhance methemoglobinemia induced by administered sodium nitrite. Nitrates do not appear to be reduced to nitrites either in the food or

TABLE 3

High dosed nitrite[1]-induced methemoglobinemia of guinea pigs fed high and low nitrate diets

Diets	Hemoglobin	Methemoglobin	Mortality[3]
	g/100 ml	*% of hemoglobin*	
High nitrate beet	12.2±0.8[2]	59.8±2.2	4/8
Low nitrate beet	12.3±0.8	60.4±2.7	2/10
High nitrate purified	12.4±0.6	62.1±2.1	4/9
Low nitrate purified	11.7±0.7	61.3±2.3	6/10

[1] 22.8 mg nitrite-nitrogen per kilogram body weight. [2] Averages ±SEM. [3] Number died/number dosed.

TABLE 4

Effect of dietary ascorbic acid and methionine on nitrite-induced methemoglobinemia[1]

Treatments			
Ascorbic acid	Methionine	Hemoglobin	Methemoglobin
%	%	*g/100 ml*	*% of Hb*
0	0	9.5±0.4[a2]	11.3±1.2[a]
0	1.0	9.1±0.8[a]	10.1±1.0[a]
0.02	0	11.6±0.9[b]	15.2±1.3[b]
0.02	1.0	12.3±0.9[b]	11.0±1.4[a]
1.0	0	12.0±0.6[b]	11.7±1.1[a]
1.0	1.0	13.8±0.4[b]	4.9±0.9[c]

[1] Two hours post dose; stomach intubated; 7.6 mg/kg nitrate-nitrogen; 7 to 8 animals per treatment. [2] Averages ±SEM. Superscript designation as described in table 2.

in vivo, as, indeed, the high nitrate diets per se did not induce methemoglobinemia. Kilgore et al. (24) had shown that rats and rabbits fed sodium nitrate or high nitrate-containing plant foods developed slightly increased blood methemoglobin levels. The possibility of in vivo nitrate reduction to nitrite in the animals these investigators used could account for this observation. Various aromatic amino and nitro compounds, which do not form methemoglobin in vitro, will do so in vivo depending on the animal species (25). It appears that in our studies, through some unknown mechanism, nitrate is synergistic to the capacity of nitrite to oxidize hemoglobin into methemoglobin of the guinea pig.

Lowered levels of blood methemoglobin, or lower mortality (tables 2 and 3), depending on nitrite intake, occurred in guinea pigs fed the low nitrate beets. It has been known since the investigations of Neill in 1925, that plant tissue has both an oxidation and reduction effect on hemoglobin (26). Natural reducing compounds in plant tissue could account for a lowered blood methemoglobin in guinea pigs fed the table beets when the nitrite intake is low or moderate. As observed in table 2, unexplained low hemoglobin levels occurred in the guinea pigs fed the high nitrate purified diet and dosed with the low concentration of sodium nitrite. The mean methemoglobin level of these animals, however, was similar to the guinea pigs fed the high nitrate beet diet and dosed with the equivalent level of nitrite.

Ascorbic acid has been used therapeutically in human cases of hereditary methemoglobinemias (27), and Koeiba and Sleight (11) observed that subcutaneous administration of nitrite to scorbutic guinea pigs produced significantly higher levels of blood methemoglobin, as compared with normal controls. Kilgore et al. (12) and Bolyai et al. (13) did not observe this relationship of methemoglobin reduction by ascorbic acid administration to guinea pigs. The freeze-dried beets contributed 108 ppm of ascorbates to the total beet diet. Although methionine levels in the high and low nitrate-containing beets were 0.68% and 0.42%, respectively, on a dry matter basis, additional dietary methionine added to a deficient diet may affect the level of methemoglobin (14) perhaps through the changed circulating level of glutathione (28) and/or ergothionine (29).

Experiment 2 was undertaken to observe the possible influence of dietary ascorbic acid and methionine on nitrite-induced methemoglobinemia. Our results, in agree-

TABLE 5

Effect of high dietary supplements of ascorbic acid and methionine on nitrite-induced methemoglobinemia, blood ascorbic acid, hemoglobin and body weight[1]

Treatments					
Ascorbic acid	Methionine	Hemoglobin	Methemoglobin	Blood ascorbic acid	Body weight
%	%	*g/100 ml*	*% of Hb*	*mg/100 ml*	*g*
0	0	10.4±0.8	16.6±1.6[a2]	0.52±0.09[a]	183± 7[a]
1.0	1.0	12.9±0.5	6.8±1.5[b]	2.44±0.42[b]	260±16[b]
2.0	2.0	12.0±0.7	5.3±0.8[b]	3.66±0.32[c]	236±13[b]

[1] One hour post dose; per os; 7.6 mg/kg nitrate-nitrogen; 10 to 12 animals per treatment. [2] Averages ±SEM. Superscript designation as described in table 2.

ment with Kilgore et al. (12) and Bolyai et al. (13), indicate that ascorbic acid alone does not appear to have a complete reducing effect on methemoglobinemia in guinea pigs. A possible explanation for these results is that ascorbic acid, so ubiquitous in its reducing or antioxidant action on cells usually in conjunction with other nutrients (30), requires dietary methionine for a rapid reduction of nitrite-induced methemoglobin. As seen in table 4, the 50-fold increase in dietary ascorbic acid, without additional methionine, brought a significant ($P < 0.05$) decrease in methemoglobin levels. Yet combination of the high dietary levels of ascorbic acid and methionine, as seen in tables 4 and 5, brought a further decrease of over 50% in blood methemoglobin values. These data support a synergistic mechanism between ascorbic acid and methionine in reducing nitrite-induced methemoglobin. These results suggest that the investigations of Kociba and Sleight (11) may have had a similar dietary interaction, although methemoglobinemia depression in guinea pigs was only attributed to the injected ascorbate in their study.

These results, of course, do not specify why nitrite-induced methemoglobinema is lowered in guinea pigs consuming beets containing low amounts of nitrate, or increased by high levels of dietary nitrates. These data do indicate that, either by direct or indirect reducing mechanisms, dietary constituents of foods can reduce methemoglobinemia and mortality developed by nitrites. This study also suggests that naturally occurring high nitrate-containing vegetables can exacerbate the toxic effect of nitrite ingestion.

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A Comparison of Two Colorimetric Methods for Determining Reduced Ascorbic Acid in Frozen Peas [a,b]

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SUMMARY

The sodium 2,6-dichlorobenzenoneindophenol method (Method A) and the diazotized 4-methoxy-2-nitroaniline method (Method B) for measuring reduced ascorbic acid in frozen peas were compared. A randomized incomplete block design with 12 replications for each of the two methods of ascorbic acid analysis was used. Very highly significant differences in ascorbic acid values were obtained between Methods A and B, with Method A always the higher.

The retention of reduced ascorbic acid in fruits and vegetables has been one criterion of quality. A number of methods have been used to measure reduced ascorbic acid. Since no one method appeared to be entirely satisfactory for measuring reduced ascorbic acid, it seemed desirable to compare two methods considered applicable to a large number of routine laboratory analyses.

The sodium 2,6-dichlorobenzenoneindophenol method (Loeffler and Ponting, 1942) depends directly on an oxidation-reduction reaction, whereas Schmall *et al.* (1953) suggested that the diazotized 4-methoxy-2-nitroaniline method is highly specific for ascorbic acid, and depends somewhat on the enediol character of ascorbic acid rather than directly on its reducing power. An apparent advantage of the diazotized 4-methoxy-2-nitroaniline method was the longer time allowance of 10 min for reading the Klett-Summerson photoelectric colorimeter after all reagents had been combined. With the sodium 2,6-dichlorobenzenoneindophenol method, readings must be made within 15 sec after the dye and acid are combined.

The purpose of the present investigation was: 1) to compare values for reduced ascorbic acid in individually frozen peas as determined by the sodium 2,6-dichlorobenzenoneindophenol and the diazotized 4-methoxy-2-nitroaniline methods, and 2) to determine any variations that exist in the quantity of reduced ascorbic acid within one lot of grade A frozen peas.

EXPERIMENTAL PROCEDURE

A randomized incomplete block design, with 12 replications for each method of ascorbic acid analysis was used. Data were analyzed by analysis of variance; Fisher's test (Fisher, 1949) for least significant difference, when appropriate; the t-test; and the Bartlett test (Snedecor, 1956).

Six 2-lb packages from one lot of U. S. Grade A fancy individually frozen peas were purchased from a local market in midsummer. Each two-pound package was gently tapped to separate the peas, and the contents emptied into a plastic bowl for thorough mixing. From the bowl, 100-g samples of peas were weighed into polyethylene bags marked to indicate the number of the 2-lb package, number of the sample, method of analysis, date of analysis, and time of analysis (morning or afternoon). Weighing was done rapidly to avoid thawing. All samples from each 2-lb package were closed, tied with "Twist-em" covered wire, and stored on one shelf of a home freezer.

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A thermostatically controlled gas burner was used to cook the frozen peas. Each sample was dropped into 80 ml of boiling distilled water in a 1-qt aluminum saucepan with a vented lid. When the water returned to a boil, the peas were cooked for 1 min with the temperature control at the highest point. Vigorous boiling was reduced by adjusting the temperature control to between 200 and 225°F, and the cooking continued for 6 min. Immediately after cooking, the peas and remaining cooking liquid were analyzed for reduced ascorbic acid by two methods. Method A was a modification of the sodium 2,6-dichlorobenzenoneindophenol method of Loeffler and Ponting (1942), and Method B was the diazotized 4-methoxy-2-nitroaniline method reported by Schmall *et al.* (1953).

The modifications of the method of Loeffler and Ponting (1942) were: 1) an increase in the size of the sample from 25 to 50 g to 100 g; 2) the addition of 5 ml of diluted filtrate to 5 ml of dye instead of adding 9 ml of dye to 1 ml of filtrate; 3) the substitution of a Klett-Summerson photoelectric colorimeter for the Evelyn photoelectric colorimeter; and 4) the use of the following formula for calculating the ascorbic acid factor.

$$\text{ascorbic acid factor} = \frac{\text{concentration of ascorbic acid in dilution}}{\text{blank reading} - \text{ascorbic acid reading}}$$

The factor, then, was used in calculating the reduced ascorbic acid concentration of the unknown solution as follows:

$$\frac{\text{ascorbic acid factor} \times \text{corrected unknown reading (blank reading} - \text{unknown reading)}}{\text{aliquot portion}} \times \text{dilution} = \text{mg reduced ascorbic acid/100 g sample}$$

RESULTS AND DISCUSSION

The investigation was completed within 7 weeks after the peas were purchased and stored. The ascorbic acid values of the peas were subjected to analysis of variance (Table 1). When appropriate, least significant difference (Fisher, 1949) was calculated.

Differences among packages were nonsignificant (Table 1), whereas differences attributable to method of analysis and the interaction of packages × methods were respectively significant at the 0.1% and 5.0% levels. The significant differences attributable to the interaction of packages × methods probably occurred in package V (Table 2). The mean value for package V analyzed by Method B was significantly higher than that for any other package analyzed by Method B; whereas the mean value for package V analyzed by Method A was significantly lower than for either package III or IV. The lowest mean value for all packages analyzed by Method A was obtained from package V.

All packages contained peas that varied widely in size from large mature to small, less mature. Ascorbic acid values that occurred within and among packages, analyzed by a given method, are illustrated in Figs. 1 and 2. According to the Bartlett test described by Snedecor (1956), the sampling variances within the same treatment and package were homogeneous. For individual samples analyzed by the sodium 2,6-dichlorobenzenoneindophenol method (Method A), the ascorbic acid values ranged from 15.77 mg/100 g peas to 17.35 mg/100 g. A range of 11.25 mg/100 g to 13.75 mg/100 g was found with the diazotized 4-methoxy-2-

Table 1. Analysis of variance of reduced ascorbic acid in peas as determined by two methods.

Source of variation	D/F	Ms and significance
Packages	5	0.2704
Methods	1	221.2***
Packages × methods	36	0.9921*
Remainder	36	0.1886
Total	47	

Table 2. Mean ascorbic acid values of methods with the interaction of packages × methods as the source of variation.

Packages	Methods: A (mg/100 g peas)	Methods: B (mg/100 g peas)
I	16.49	11.93
II	16.76	12.34
III	16.96	12.11
IV	17.09	12.21
V	16.17	13.18
VI	16.36	12.29
	lsd* = 0.62	

* A = sodium 2,6-dichlorobenzenoneindophenol method; B = diazotized 4-methoxy-2-nitroaniline method; lsd* = least significant difference at the 5.0% level.

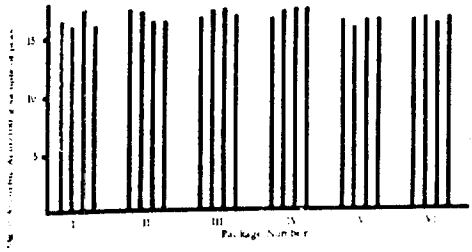

Fig. 1. Ascorbic acid values of 100-g pea samples within a package analyzed by Method A (sodium 2,6-dichlorobenzenoneindophenol method).

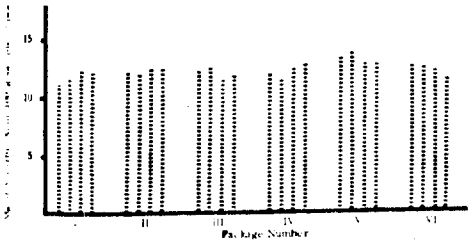

Fig. 2. Ascorbic acid values of 100-g pea samples within a package analyzed by Method B (diazotized 4-methoxy-2-nitroaniline method).

nitroaniline method (Method B). The values found with Method A were closer than the values found with Method B to the average values reported by Burger *et al.* (1956) for frozen green sweet peas (17.1 mg/100 g) and by Watt *et al.* (1950) and Bowes and Church (1956) for cooked peas (15 mg/100 g) and frozen peas (18 mg/100 g). Therefore, it appeared that Method A most nearly reflected the reduced ascorbic acid concentration of the peas analyzed and would be the method recommended for subsequent analyses. Table 2 shows variations in mean ascorbic acid values for packages of one lot of U. S. Grade A Fancy peas analyzed by each method.

Although the differences in reduced ascorbic acid among individual samples of peas seemed small (Figs. 1 and 2), some of the mean values (Table 2) were significantly ($P < .05$) different from each other. With Method A the mean ascorbic acid value was significantly greater for package IV than for packages V and VI. Package III also had significantly more ascorbic acid than package V. All other differences among packages analyzed by Method A were nonsignificant. The Method B analysis indicated that package V contained significantly more ascorbic acid than any other package and that differences among the other packages were nonsignificant.

The interaction of packages × methods was significant; packages and methods were confounded with the time of analysis. Therefore, t-tests were made to determine if there were significant variations in ascorbic acid values. Consideration of each method separately resulted in no difference attributable to the time of analysis. When Method A was compared with Method B, and when the analyses done in the morning and the afternoon were considered separately, Method A gave decidedly higher numerical values than Method B at each time of day.

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Ascorbic Acid Depletion in Patients Undergoing Chronic Hemodialysis[1]

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THE DIET CONSUMED by patients with chronic renal failure is restricted in protein to reduce accumulation of nitrogenous metabolites (1, 2). The dietary content of sodium and potassium is also limited in an effort to prevent development of hypertension and hyperkalemia. Because of these restrictions, only small quantities of citrus fruits and leafy vegetables are ingested, hence, the intake of certain water soluble vitamins (thiamin, niacin, B_6, folic acid, riboflavin, pantothenic acid, and ascorbic acid) is low. Some physicians advise their patients that green vegetables should be treated by prolonged boiling to reduce the potassium content before they are eaten (3). Since ascorbic acid is heat labile and boiling removes the B vitamins from vegetables, intake of these nutrients will be further diminished by this treatment.

Folic acid deficiency, manifested by low plasma folate levels and megaloblastic changes in the bone marrow, has been found in some uremic patients (4). Folate depletion probably results from both low intake and loss of the vitamin during hemodialysis (5, 6). In contrast to folic acid, the plasma concentration of vitamin B_{12} is normal or elevated in patients with chronic renal insufficiency (5). Studies concerning the effect of hemodialysis on plasma levels of other B complex vitamins have provided conflicting data (7–9). Few investigations concerning the effect of hemodialysis on plasma ascorbate have been reported although several workers have mentioned the need to measure vitamin C levels (8, 9). Haslik (10) observed that in patients with acute renal failure, hemodialysis resulted in a reduction of plasma vitamin C. However, he did not investigate plasma ascorbate levels of patients with chronic renal failure receiving maintenance hemodialysis therapy.

The vitamin C intake of patients receiving chronic hemodialysis varies depending on the prescribed diet, frequency of dialysis, and composition of the dialyzing fluid. If the potassium concentration in the dialysate is low, the physician may allow the patient to take more potassium-containing foods, e.g., citrus fruits. Pendras and Erickson (11) reported that three of five chronically dialyzed patients had an adequate intake of vitamin C, but as some water soluble vitamins are dialyzable (9), there is a need for vitamin supplementation. The study reported here was carried out in order to determine the quantity of ascorbic acid lost by patients undergoing chronic hemodialysis and to assess the need for ascorbic acid supplementation.

METHODS

Sixteen patients with chronic renal failure who had been treated with maintenance hemodialysis for periods of 4 months–4 years were

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selected for study. The patients received hemodialysis for 6–8 hr at least twice weekly, depending on their body weight and clinical condition.

Equipment

The Ultra-Flow 100 twin coil dialyzer (PT 150 cuprophan membrane) with standard 100-liter tank was used and the dialysate fluid was prepared from Travenol dialysate concentrate that contained no ascorbic acid. When diluted to 100 liters, solute concentrations in the dialysate were: calcium = 2.5 mEq/liter, dextrose = 250 mg/100 ml, magnesium = 1.5 mEq/liter, potassium = 2 mEq/liter, acetate = 37 mEq/liter, sodium = 130 mEq/liter, and chloride = 99 mEq/liter. The tank was drained and fresh dialysate fluid was added at the halfway point (3–4 hr) of each dialysis.

Diet

All patients entering the hemodialysis program were requested to follow a 60-g protein, 400-mg sodium, 1,500-mg potassium diet that provided approximately 2,000 kcal. They were given a printed diet with instructions, sample menus, and exchange lists. Initially, a dietitian spoke with each patient and his family concerning preparation of meals. Thereafter, periodic diet reviews with each patient were conducted by the dialysis nursing staff. Patients were advised to limit their fluid intake to 1,000 ml/24 hr plus an amount equal to their 24-hr urine volume.

When the present study was undertaken, it was desired to determine as accurately as possible the quantity of ascorbic acid that the patient consumed in his diet. To estimate dietary intake of ascorbic acid, patients were given a list of foods known to contain moderate or high levels of vitamin C. They were asked to keep a record of foods consumed during a week and the approximate quantity eaten. From these dietary histories, an estimate of the amount of ascorbic acid ingested was made, using a Department of Agriculture handbook on the vitamin C content of foods (12).

Medications

All patients were placed on aluminum hydroxide gel to prevent excessive absorption of phosphorus. Three patients received oral iron therapy because of low serum iron levels. Patients rarely needed blood transfusions, the average transfusion rate for all chronically dialyzed patients being 0.22 units packed red cells/patient per month.

Plan of the Study

After carefully explaining to each patient the purpose of the study and obtaining consent for participation, supplementary vitamin therapy was discontinued for at least 3 months prior to beginning the investigation.

Patient groups. Eleven patients received no ascorbic acid during the entire period of study (5 months). To evaluate the effect of regular hemodialysis therapy in patients who were not receiving vitamin C, plasma samples for ascorbate levels were obtained at 7- to 21-day intervals.

Five patients were given 250–500 mg oral ascorbic acid daily during the first 2 months of the study. Vitamin C treatment was then stopped and the patients followed for 3 months with plasma ascorbic acid levels being determined periodically.

Throughout the study, patients were carefully examined to detect changes in the appearance of their skin or gums.

Collection of specimens and chemical analysis. Blood specimens obtained for determination of ascorbic acid levels were collected in heparin-containing tubes and immediately centrifuged. The plasma was removed and kept frozen until analyses were performed. Aliquots of dialysate were also frozen until the vitamin C content could be determined. Ascorbic acid was determined by the method of Roe and Kuether (13). "Predialysis" specimens were obtained immediately prior to the start of hemodialysis and "postdialysis" specimens were collected at the conclusion of dialysis. Prothrombin times were determined on patients who were not receiving vitamin C.

Ascorbic acid loss into dialysate. The relationship between plasma ascorbate levels and the amount of vitamin C lost into the dialysate bath was investigated in 15 patients. A predialysis plasma sample and two aliquots of dialysate fluid were obtained each time a patient was hemodialyzed. One sample of dialysate was collected at the halfway point (before the bath change) and the second at the end of dialysis.

Vitamin C levels in the dialysis fluid were determined by the same technique used for plasma ascorbate measurement. The total amount of vitamin in the dialysis bath was calculated by multiplying the concentration of ascorbic acid (milligrams per milliliter) by the total volume of dialysate.

In vitro extraction of ascorbic acid from blood. To determine the rate of extraction of ascorbic acid from plasma by the artificial kidney, two in vitro studies were performed utilizing the twin coil dialyzer and a "reservoir" of bank blood (500 ml) to which 6 mg ascorbic acid was added. Dialysis was carried out for 1–2 hr and blood samples were assayed for vitamin C content at 10–15 min intervals.

Dialysance of ascorbic acid. Dialysance studies for ascorbic acid were performed by collecting simultaneous specimens of dialysate fluid and blood entering and leaving the dialyzer. After determination of dialysate and plasma ascorbic acid levels, the values were substituted in the dialysance formula (14).

$$D = \frac{A - V}{A - B} \times BF$$

where D = dialysance of ascorbic acid in milliliters per minute, A = concentration of ascorbic acid in blood entering dialyzer, V = concentration of ascorbic acid in blood leaving dialyzer, B = concentration of ascorbic acid in dialysate fluid, and BF = blood flow through dialyzer in milliliters per minute.

RESULTS

Patients in *group A* who did not receive itamin C supplements demonstrated a onsistent decrease in plasma ascorbic acid uring each dialysis with a mean decline f 40 ± 4% from the predialysis value Fig. 1). During the 5-month period of obervation, there was a progressive decline f plasma ascorbate levels in individuals who did not receive ascorbic acid. In five patients, plasma ascorbate values fell to levels between 0.05 and 0.14 mg/100 ml (normal values for plasma or serum ascorbic acid range from 0.4 to 1.5 mg/100 ml). Vitamin C supplementation was resumed in these individuals to prevent the possible development of early scurvy.

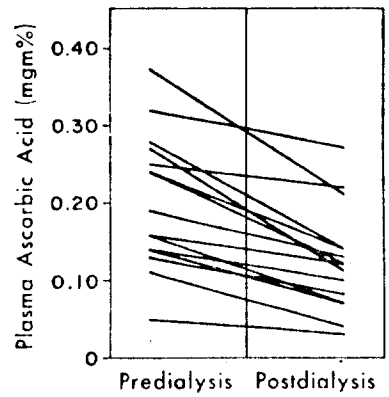

FIG. 1. Effect of hemodialysis on plasma ascorbic acid levels in patients not receiving vitamin C supplements. Each line represents the dialysis of one patient and plasma ascorbic acid levels immediately before and after dialysis.

Large oral doses of vitamin C administered to five patients (*group B*) resulted in marked elevation of plasma ascorbate levels, although the values decreased rapidly when vitamin therapy was discontinued (Table 1). The mean reduction of plasma ascorbic acid after 6–8 hr of hemodialysis in patients receiving vitamin C supplements was 68 ± 4%. Loss of ascorbic acid during dialysis was especially great when plasma concentrations were at their highest values. In one patient with a predialysis plasma ascorbate level of 11.77 mg/100 ml, 780 mg vitamin C were found in the dialysate.

In vitro studies, incorporating a closed circuit with a reservoir of ascorbic acid-enriched blood, demonstrated a 75–90% decline in the plasma ascorbate level within 20 min (Fig. 2). At the end of 40–45 min of dialysis at a blood flow rate of 265–285 ml/min, plasma ascorbic acid and dialysate ascorbate levels equilibrated in one instance. In a second study, plasma ascorbate levels were too low to be detected by assay after 40 min.

Dialysance studies revealed that vitamin C was continuously extracted from blood throughout dialysis. Dialysance appeared to increase with the rate of blood flow,

TABLE I

Plasma ascorbic acid levels in hemodialyzed patients receiving vitamin C supplements, mg/100 ml

Name	Date	Predialysis	Post-dialysis	% Decline
TM[a]	4/11	1.96	0.67	65
	4/16	1.51	0.23	80
	4/28	2.53	0.62	75
VR[b]	4/10[c]	15.30	3.24	78
	4/17	0.90	0.30	66
	4/24	0.67	0.35	47
	5/8	0.45	0.27	40
FF[b]	4/10	10.24	1.61	84
	4/29[d]	2.66	0.45	83
NI[a]	4/10	3.56	1.11	69
	5/1[e]	0.87	0.32	63
JG	4/11	1.23	0.42	65

[a] Received 250 mg ascorbic acid twice daily for 2 months. [b] Received 500 mg ascorbic acid twice daily for 2 months. [c] Ascorbic acid discontinued 4/7. [d] Ascorbic acid discontinued 4/17. [e] Ascorbic acid discontinued 4/29.

although the vitamin was more slowly cleared than urea or creatinine (Fig. 3). During 15 individual dialyses, the total amount of vitamin C that appeared in the dialysate varied from 80 to 280 mg (Fig. 4). Predialysis plasma ascorbate levels in these patients ranged from 0.10 to 2.26 mg/100 ml (with 10 values of less than 0.51 mg/100 ml).

During the course of the study two patients demonstrated skin changes consistent with those that occur in ascorbic acid deficiency. One patient developed small, raised, perifollicular, hyperkeratotic areas on the arms, back, and thighs after 4 months of receiving no vitamin C; however, no hemorrhages were noted in these areas. The plasma ascorbic acid level was 0.14 mg/100 ml at the time the lesions appeared. The hyperkeratotic areas became progressively smaller and disappeared after the patient received 50 mg ascorbic acid daily for 4 weeks. A second patient had small hemorrhagic areas on the forehead and chin, which appeared after 4 months without vitamin C supplementation. Plasma ascorbic acid level was 0.08 mg/100 ml. The patient wa

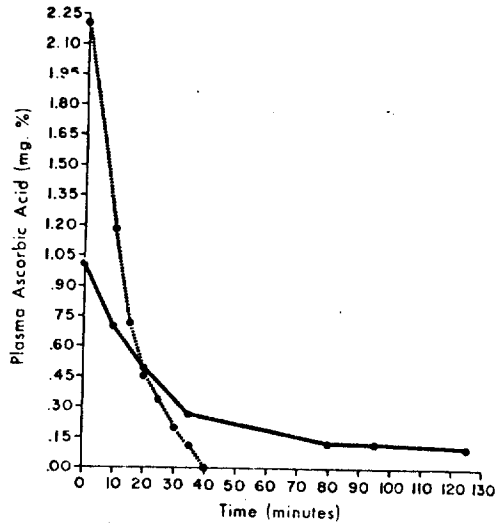

FIG. 2. Decline of plasma ascorbic acid during two in vitro dialyses, utilizing the twin coil.

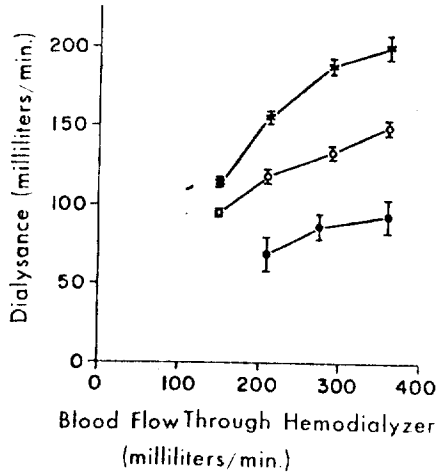

FIG. 3. Comparative dialysance values of ascorbic acid, urea, and creatinine at varying blood flows (mean ± standard error of mean). Dots represent mean dialysance of ascorbic acid in six patients, open circles represent mean dialysance of creatinine in 10 patients, and X's represent mean dialysance of urea in 10 patients.

placed on 125 mg ascorbic acid daily and the lesions disappeared in 2 weeks. Two patients with plasma ascorbic acid levels less than 0.13 mg/100 ml demonstrated prolonged prothrombin times. In one of the patients, swelling of the parotid and submandibular glands developed. Both patients were treated with 125 mg ascorbic acid/day. Within 2 weeks, swelling of the salivary glands disappeared, and after 4 weeks, prothrombin times had returned to normal.

Dietary histories revealed that the mean intake of ascorbic acid was 34 ± 4 mg/day during the 7-day period of study. Potatoes and lettuce appeared to be the main sources of vitamin C.

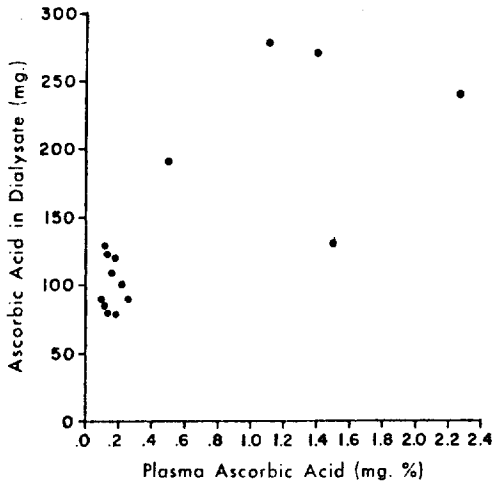

FIG. 4. Plasma ascorbate levels and ascorbic acid loss into dialysate during 15 dialyses. Each dot represents the total amount of ascorbic acid recovered from the dialysate during a single dialysis.

DISCUSSION

The studies reported here demonstrate that during hemodialysis there is a significant decline in plasma ascorbic acid. In patients who were not receiving vitamin C, the mean decrease of plasma ascorbate was 40 ± 4%. The continued loss of ascorbic acid by patients dialyzed twice weekly resulted in a progressive reduction of plasma vitamin C. In five patients, ascorbate concentrations declined to such low levels that we felt it was necessary to administer vitamin C supplements to prevent the development of scurvy. Despite predialysis plasma ascorbic acid values of less than 0.10 mg/100 ml in several patients, the levels were even further reduced after a 6- to 8-hr period of hemodialysis. Patients who received large oral doses of vitamin C (250–500 mg/day) had high plasma ascorbic acid levels. When these patients were dialyzed, the plasma ascorbate concentration declined by 68 ± 4%. The actual amount of vitamin C lost from the blood of supplemented patients was many times greater than that of the nonsupplemented group since predialysis plasma vitamin levels were higher and the percent decline greater during hemodialysis in those receiving ascorbic acid. An interesting and important observation was that in two patients with plasma ascorbate concentrations of 0.06 mg/100 ml and 0.08 mg/100 ml, the administration of 50–125 mg vitamin C daily, over a 2-month period, failed to increase the plasma concentration of the vitamin to more than 0.16 mg/100 ml. The lack of response suggests that this amount of ascorbic acid is not sufficient to replace the losses that occur during hemodialysis.

In two in vitro experiments, measured amounts of ascorbic acid were added to blood that was placed in the artificial kidney and dialyzed. The plasma vitamin C concentration declined more rapidly in vitro (70–90% reduction in 30 min) than in vivo, so that within 2 hr almost all the vitamin was removed from the blood. The most likely explanation for the slower loss of ascorbic acid from the blood of the dialyzed patient under in vivo conditions is that in the human subject, plasma vitamin C is in equilibrium with tissue ascorbic acid. When the vitamin is removed from blood by hemodialysis it is replaced by ascorbic acid released from various tissue stores. If this process is continued

over a prolonged period and the intake of vitamin C is restricted, the tissue content of ascorbic acid will be depleted. In future studies, tissue ascorbate content of patients undergoing hemodialysis will be determined by measuring the concentration of the vitamin in white blood cells.

During hemodialysis, substantial amounts of ascorbic acid appeared in the dialysate. Loss of vitamin C continued even though plasma ascorbate levels were low. In one patient whose plasma vitamin C concentration was 0.11 mg/100 ml, 130 mg ascorbate were recovered from the dialysate. When plasma vitamin C levels were higher, even larger amounts of the vitamin traversed the dialysis membrane (up to 780 mg with a plasma level of 11.77 mg/100 ml). The quantity of ascorbic acid lost by patients undergoing dialysis makes it evident that vitamin C deficiency will develop unless body stores of the vitamin are replenished.

Vitamin C, urea, and creatinine are continuously removed from plasma during hemodialysis although the dialysance (or clearance) rate of ascorbic acid is less than that of urea or creatinine. Even though vitamin C is cleared from the plasma at a slower rate than urea or creatinine, a significant decline in plasma ascorbic acid levels occurs in the patient undergoing maintenance hemodialysis. These observations may be explained by the fact that in uremic patients, plasma ascorbate levels are normal or depressed, whereas blood concentrations of nitrogenous substances are elevated. When patients with terminal renal disease become oliguric, serum potassium values rise because of diminished urinary excretion of the cation. For this reason they are instructed to eat limited quantities of citrus fruits, tomatoes, and green, leafy vegetables because of the high potassium content. Food products that contain large amounts of potassium are also rich in vitamin C (15). Thus, if potassium-restricted diets are followed for extended periods, a significant cumulative deficit of ascorbic acid may result. Our patients demonstrated the low intake of ascorbic acid that results from dietary restriction since the mean intake of vitamin C was 34 ± 4 mg/day during the week that dietary histories were recorded. The recommended daily dietary allowance for normal adults in the United States is 55–60 mg per day (16), although the British Medical Research Council claims that a daily uptake of 10 mg ascorbic acid is sufficient, both to prevent scurvy over a prolonged time and to cure scurvy once it has developed (17). Thus our patients not only lost large amounts of ascorbic acid during hemodialysis but their intake of vitamin C was reduced. These facts explain the progressive decline of serum ascorbate levels that was observed in patients who were not given supplementary vitamin C. Unless patients on long-term hemodialysis therapy receive vitamin C supplements or are given more liberal diets, loss of ascorbate by dialysis will probably always exceed dietary intake. Unlike folic acid or the essential amino acids that are removed from plasma during hemodialysis, dietary replenishment of vitamin C is inadequate (5, 18, 19).

Clinical scurvy has not been reported in patients undergoing maintenance hemodialysis. In the present investigation three patients whose plasma ascorbate levels were 0.13 mg/100 ml or less demonstrated clinical findings compatible with the early stages of vitamin C depletion (hyperkeratotic skin lesions and swollen parotid and submandibular glands) (20). These findings disappeared following administration of small oral supplements of ascorbic acid (50–125 mg/day) for 3 weeks. In addition, two patients developed prolonged prothrombin times that returned to normal with ascorbic acid supplementation. It has been reported (21) that with the onset of scurvy, an impairment in the clotting mechanism may occur, as reflected by a prolonged prothrom-

bin time. Recently Hodges et al. (22) reported that at very low plasma ascorbic acid levels, the biochemical method of detecting the vitamin is not reliable. Chromatographic studies demonstrated that some of the reacting substances were not ascorbic acid. Thus, it is possible that ascorbic acid levels in some of our patients were even lower than indicated by the chemical procedure used to measure vitamin C. This may account for the fact that clinical scurvy has been reported in patients with measurable amounts of ascorbic acid in their plasma (23). Plasma ascorbate levels reflect recent intake of vitamin C, whereas white blood cell ascorbic acid concentration is a better indicator of tissue vitamin C stores (24). In our future studies, ascorbic acid will be determined in leukocytes as well as in plasma so that a more accurate assessment of vitamin C stores can be made. Uremia and clinical scurvy have a number of common clinical findings (anemia, poor tissue healing, bleeding diatheses, coagulation defects, skin changes, et cetera). The possibility exists that some of the residual effects of uremia noted even in well-dialyzed patients (anemia, occasional poor wound healing, increased susceptibility to infection) may be a result of severe vitamin C depletion.

Ascorbic acid supplementation is an essential part of the therapeutic regimen in patients undergoing maintenance hemodialysis. Determination of ascorbic acid loss during a single hemodialysis (70–90 mg in nonsupplemented patients) and decreased consumption of vitamin C in the diet (less than 40 mg/day) suggest that these patients be given daily ascorbic acid supplements of 150–200 mg/day. Patients who receive more frequent hemodialysis or consume a more restricted diet will require greater supplementation. The addition of a minute amount of ascorbic acid to the hemodialysate concentrate, establishing a dialysate level of 0.4–0.6 mg/100 ml would probably prevent excessive removal of vitamin C from the plasma of dialyzed patients. When the frequency of dialysis is increased to three or more dialyses per week, the danger of hyperkalemia is less and the patient's diet should be cautiously expanded to allow a more liberal intake of potassium-containing foods, such as green vegetables. In this manner, the vitamin C content of the diet will be increased.

SUMMARY

Ascorbic acid is removed from the plasma of patients undergoing hemodialysis. Patients with chronic renal disease who were not receiving vitamin C supplementation demonstrated a mean decline in plasma ascorbic acid of 40 ± 4% during hemodialysis, whereas the mean decline in patients receiving vitamin C supplements was 68 ± 4%. Removal of vitamin C from the plasma continued despite a marked decline in plasma ascorbate in nonsupplemented patients. Dietary histories revealed that ascorbic acid intake of the patients was 34 ± 4 mg/day. Since the ascorbic acid lost from the plasma during dialysis is not adequately replaced by dietary consumption of vitamin C, patients undergoing maintenance hemodialysis should receive ascorbic acid supplementation as an important part of their therapeutic regimen.

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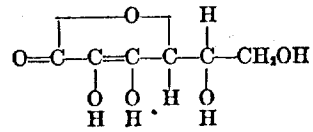
A HIGHLY SPECIFIC PROCEDURE FOR ASCORBIC ACID*

By M. X. SULLIVAN and H. C. N. CLARKE† (Georgetown University, Washington, D. C.)

For the estimation of the antiscorbutic factor, ascorbic acid or vitamin C, in foodstuffs, tissues, etc., many methods have been employed, all of which depend on the strong reducing capacity of the vitamin. Ehrlich (1) in 1886 noted that some tissue would not reduce indophenol or alizarin blue, that other tissues would reduce the indophenol but not alizarin blue, while a few (lungs, liver, and mucosa of stomach) would reduce the difficultly reducible alizarin blue. In 1895 Smith (2) reported a peculiar hemorrhagic condition in guinea pigs that had been restricted to a cereal diet without grass, clover, or succulent vegetables. The importance of this observation was not appreciated until 1912 when Holst and Frölich (3) pointed out the similarity between this disease in guinea pigs and scurvy in man. By 1918 it was well recognized that scurvy was a deficiency disease (4–6). In 1919, Drummond (7) designated the antiscorbutic substance as "Vitamin C."

In 1928 Szent-Györgyi (8) isolated a reducing substance from the adrenal cortex and from various plant sources. He gave it the formula $C_6H_8O_6$ and thought he had a hexuronic acid. Then, independently, Waugh and King (9) isolated the crystalline antiscorbutic substance, and about the same time, and again independently, Svirbely and Szent-Györgyi (10) recognized the identity of the hexuronic acid with vitamin C. Haworth and Szent-Györgyi (11) named the substance ascorbic acid, and Herbert and associates (12) gave it the accepted formula:



In 1911, while studying oxidation and reduction in plants and soils, Schreiner and Sullivan (13) came to the conclusion that the power of plants to reduce dyestuffs and other reducible compounds was due to unsaturated organic material. At that time some attention was given to Ehrlich's alizarin blue findings but with no satisfactory results. Some years later Nelson and Browne (14), following the procedure of Winter (15) on invert sugar, isolated a material of high reducing power from dextrose treated with calcium hydroxide; this material was first named glucic

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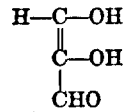
acid by Dumas (16) and was subsequently named reductone by von Euler and Martius (17) who proved that it had the structure:



Since reductone, although inactive physiologically, reacts in practically every chemical test recommended for the determination of ascorbic acid (iodine titration, 2,6-dichloroindophenol, and phenylhydrazine) a procedure was sought in which reductone does not interfere significantly with the determination of ascorbic acid.

Such a procedure is presented here. It depends on the reduction of ferric chloride by ascorbic acid, the estimation of the ferrous iron by α,α'-dipyridyl, and inhibition of other reducers by the presence of orthophosphoric acid and a high degree of acidity (*p*H 1–2).

The use of α,α'-dipyridyl for the determination of ferrous iron was introduced by Hill (18). Albert and Gledhill (19) found α,α'-dipyridyl to be highly specific for ferrous iron.

In 1936 McFarlane (20) made a study of the relative capacities of tissue extracts to reduce ferric iron and 2,6-dichloroindophenol, respectively. He employed dipyridyl to determine the quantity of ferrous iron produced when ferric iron was reduced by the biological material, and he computed the amount of ascorbic acid required for the reduction. McFarlane demonstrated that the amount of ascorbic acid determined was always lower than that obtained by the dye titration. However, he did not consider the reductone which would be estimated by his procedure.

Von Euler and Martius found that reductone has a reducing capacity twice that of ascorbic acid as determined by 2,6-dichloroindophenol. Miller (21), by a formaldehyde procedure, found reductone present in all processed foods studied; the foods included preserved foods, honey, canned concentrates, and dehydrated foods. Rehberg and Lindberg (22) emphasized that part of the indophenol-reducing substances normally excreted originates from roasted matter in the diet.

In the early work on the differentiation of vitamin C from reductone, a reductone solution was made according to Kertesz (23) as follows: Five ml of a 0.5% glucose solution and 0.5 ml of normal NaOH were placed in a test tube closed by a Bunsen valve. The mixture was heated at 80°C. for 12 minutes and was then cooled and acidified with 1 ml of 10% HCl. According to Kertesz, 1 ml of the reaction mixture equals 0.25 mg of ascorbic acid in its action on 2,6-dichlorindophenol. It was tested in the dipyridyl reaction against ascorbic acid and other reducing compounds. Thus to 1 ml of each of the following substances were added 1 ml of 3% $FeCl_3$ and 5 ml of 0.1% α,α'-dipyridyl:

Ascorbic acid (0.05 g/100 ml H_2O)	immediate pink color
Glucoreductone solution	immediate pink color
Glutathione (0.1 g/100 ml H_2O)	no color in 30 minutes
Cysteine · HCl (0.1 g/100 ml H_2O)	no color in 30 minutes
Glyoxal (0.1% aqueous solution)	no color in 30 minutes
Methyl glyoxal (40% solution)	no color in 30 minutes
Acetol (50% solution in MeOH)	no color in 30 minutes
Uric acid (0.1 g/100 ml H_2O)	no color in 30 minutes
Creatinine (0.1 g/100 ml H_2O)	no color in 30 minutes

Since reductone was the only one of the reducing substances that gave an immediate reaction like ascorbic acid, attempts were made to eliminate the reductone. After some success with other relatively tedious procedures, we tried orthophosphoric acid (employed by Snell and Snell (24) to eliminate a number of interfering substances in the determination of iron by means of α,α'-dipyridyl). When a sufficient amount of orthophosphoric acid was used, it entirely inhibited reductone, and though it retarded the action of ascorbic acid somewhat, a very sensitive reaction for ascorbic acid was still obtained. Neither glucosone (25) nor reductic acid (26) gave a positive test by this procedure. For the reductone work we used a lead salt prepared according to von Euler and Martius (17), since the reductone lead salt was quite stable. Reductone without phosphoric acid was strongly positive but it was negative in the presence of the phosphoric acid. These conclusions are exemplified in the following experiment in which 1 ml of 20% $FeCl_3$, freshly prepared, and 5 ml of 0.5% α,α'dipyridyl were added to each of the solutions shown in Table 1. It is noted that phosphoric acid entirely eliminates the interference of reductone.

TABLE 1.—*Effect of orthophosphoric acid*

SOLUTIONS TESTED	COLORIMETRIC READINGS (KLETT-SUMMERSON)	
	AFTER 60 MIN.	AFTER 90 MIN.
1.0 ml H_2O+0.3 ml H_3PO_4[a]	27	27
1.0 ml reductone lead salt (25 mg/100 ml H_2O; no H_3PO_4)	600	605
1.0 ml reductone lead salt (25 mg/100 ml H_2O)+0.3 ml H_3PO_4	25	26
1.0 ml ascorbic acid solution (0.125 mg) +0.3 ml H_3PO_4	219	232
1.0 ml of mixture of 1.0 ml reductone lead salt (50 mg/100 ml)+1 ml ascorbic acid solution (0.250 mg)+0.3 ml H_3PO_4	218	230

[a] 85 per cent orthophosphoric acid used in all cases.

EXPERIMENTAL

REAGENTS

(a) *Distilled water.*—H_2O (and glassware) must be free from oxidizing or reducing agents. Redistil H_2O twice out of glass.

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(b) *Orthophosphoric acid.*—85% (Merck's reagent grade or equivalent). Add 0.3 ml to test solutions to give a *p*H of 1–2.

(c) *Ferric chloride.*—1% and 20% aq. solns. Use for not longer than 1 day.

(d) *α,α'-Dipyridyl.*—Weigh 0.5 g into 50 ml H_2O in a 100 ml vol. flask. Shake while warming under a stream of hot H_2O. When the dipyridyl is dissolved, dil. to 100 ml.

(e) *Ascorbic acid standard solution.*—Prep. fresh at each determination (can be kept in refrigerator until used). The concn of the standard should be as close as possible to that of the soln being tested. For urine, dissolve 20 mg ascorbic acid in 100 ml H_2O. Dil. 10 ml of this soln to 100 ml with H_2O in a 100 ml vol. flask.

GENERAL PROCEDURE

The best procedure is as follows: To 1 ml of a soln contg ca 0.02 mg ascorbic acid are added 0.3 ml 85% H_3PO_4 to pH 1–2, 5 ml 0.5% aq. α,α'-dipyridyl, and 1 ml 1% $FeCl_3$.

APPLICATIONS

Application to urine.—A part-day sample of freshly voided urine was used. The indophenol titration gave a value of 1.369 mg ascorbic acid per 100 ml. For comparison with the dipyridyl method the following experiment was arranged (Table 2) in which 0.3 ml 85% H_3PO_4, 5 ml 5% α,α'-dipyridyl, and 1.0 ml 20% $FeCl_3$ were added to each of the solutions *except* the blanks. The α,α'-dipyridyl method gives a slightly lower value than does the indophenol titration. Added ascorbic acid is recovered quantitatively.

TABLE 2.—*Application to urine*

SOLUTIONS TESTED	READING (15 MIN.)	READING (CORR.)	ASCORBIC ACID (MG/100 ML)
1 ml urine + 7.3 ml H_2O	3	0	—
1 ml urine + 1 ml ascorbic acid solution (0.01 mg) + 6.3 ml H_2O	3	0	—
2 ml H_2O	6	0	—
1 ml ascorbic acid solution (0.01 mg) + 1 ml H_2O	52	46	1.00
1 ml urine + 1 ml H_2O	69	60	1.304
1 ml urine + 1 ml ascorbic acid solution (0.01 mg)	116	107	2.326

Application to orange juice.—The dipyridyl method was applied satisfactorily to canned orange juice that had been diluted 10 to 20 times, mixed well, and centrifuged. The method was modified by using 1% $FeCl_3$ in place of the higher concentration of the ferric salt. A sample of orange juice gave a value of 33.8 mg per 100 ml undiluted juice by the indophenol method and 33.16 mg per 100 ml by the dipyridyl method. Later, fresher samples gave higher values.

Application to honey.—Ten ml of glass-packed honey was diluted with an equal volume of water. The indophenol titration gave an average value of 1.35 mg ascorbic acid per 100 ml. The α,α'-dipyridyl procedure gave a value of 0.774. If the difference is accepted as due to reductone-like material, the present work verifies the statement of Miller (21) that honey contains reductone.

Application to tissues.—No application has yet been made to tissues, since a deproteinizing agent that would not cause interference would have to be found.

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A later paper will cover this phase of the problem; another article will compare the α,α'-dipyridyl procedure to iodine titration and indophenol, and possibly will describe a direct estimation of reductone.

SUMMARY

A highly specific test for ascorbic acid has been developed that depends on the reduction of ferric chloride in the presence of orthophosphoric acid at *p*H 1–2 and estimation of the ferrous iron by α,α'-dipyridyl. In the presence of phosphoric acid and at this *p*H, reductone, glucosone, reductic acid, and α-tocopherol do not interfere, nor do glutathione, cysteine, acetol, methyl glyoxal, or creatinine. To date the test has been applied to orange juice, honey, and urine. The test is sensitive to 5 micrograms of ascorbic acid per ml.

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Ascorbic acid esters and skin pigmentation

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It has been proved that one of the important physiological actions of vitamin C is that of formation of colloidal intercellular substance and collagen fibrils. Hemorrhaging due to poor development of the cement substance of the capillary wall may be caused by deficiency of vitamin C. The latter is also stored in the ductless glands and is closely related to the hormones secreted by these glands. Studies are also found on the reduction of resistance against bacteria due to deficiency of vitamin C, and on the relationship of vitamin C and antiallergic activity. Vitamin C is also closely related to pigmentation in the living organism.

L-Ascorbic acid has a potent reducing activity which has been used in many ways. In an aqueous solution it decomposes rather readily on longer storage with a formation of color and gas evolution, which is accelerated by heat, alkali and a wide variety of cations, e.g., iron, copper, etc. Therefore, an attempt at the formulation of drugs or cosmetics containing ascorbic acid leads to difficulties from the pharmaceutical point of view; a number of studies have been undertaken to overcome this problem.[1-20] Among others, the chemical modification of ascorbic acid, i.e., the esterification of the hydroxyl group, is of particular interest. The palmitic acid ester was first synthesized in this connection, although its chemical structure was unidentified.[21] The fatty acid ester which was esterified at the 6 position of ascorbic acid was synthesized later by Swern.[22]

Generally, the partial acylation of the enediol system would give rise to two isomers, 2- and 3-ester, both of which are markedly decreased in reducing power and therefore stabilized against oxidation. Although there are a few facts which remain to be proved on the structures at which positions the ester groups are introduced, the following ascorbic esters have been reported: ascorbic acid-3-palmitate,[23, 24] ascorbic acid-2,6-dipalmitate,[23, 25] ascorbic acid-3,6-dipalmitate,[25] and ascorbic acid-2,5,6-tripalmitate[23] as the higher fatty acid esters of ascorbic acid; and ascorbic acid-2-benzoate, 5,6-isopropylidene-ascorbic acid-3-benzoate, ascorbic acid-2,6-dibenzoate, ascorbic acid-3,6-dibenzoate, and ascorbic acid-3,5,6-tribenzoate as the organic acid esters.[26] The ascorbic acid activities of those esters tend to be lower in proportion to the increase in the number of substituents in the molecule.[27,29] On the other hand, the phosphate and sulfate esters are available as the inorganic acid esters of ascorbic acid.[30-35] The latter was isolated by extraction from brined shrimp eggs; studies related to its physiological significance are under way.[32]

Ascorbic acid 2- and 3-phenylphosphate have been synthesized by Clark, et al.[34] The preparation of ascorbic acid phosphate of unknown structure has been reported by Cutolo, et al.[33] Recently, Nomura, et al. have separated the mixed product, obtained by phosphorylation of L-ascorbic acid with phosphoryl chloride, by ion exchange chromatography into four components which have been assigned to L-ascorbic acid 3- and 2-phosphate, L-ascorbic acid 3-pyrophosphate, and bis(L-ascorbic acid 3,3-) phosphate respectively.[35] Furthermore, the preparative method useful for the industrial production of L-ascorbic acid 3-phosphate has been established by the selective phosphorylation of the hydroxyl located on the C_3 position of 5,6-isopropylidene-L-ascorbic acid.[36]

This article is based on a manuscript published in Vitamin 41, 387, 1970 (Japan).

Synthesis and chemical structures of L-ascorbic acid phosphates

Phosphorylation of 5,6-isopropylidene-L-ascorbic acid with an equivalent amount of phosphorus oxychloride and excess of base in acetone yields a mixture of four L-ascorbic acid phosphates. This can be

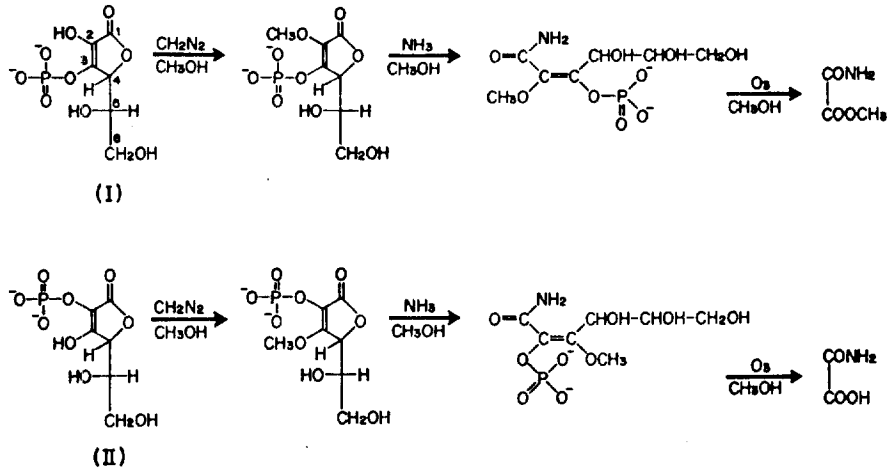

Chart 1.

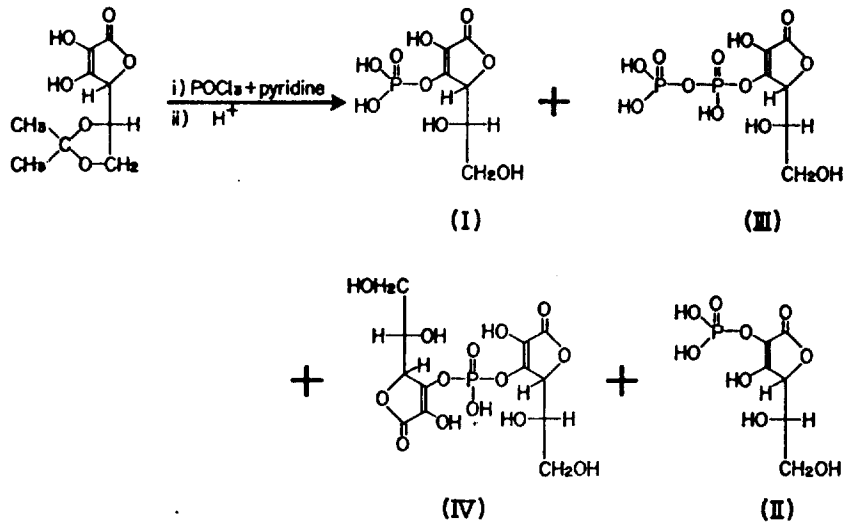

Chart 2.

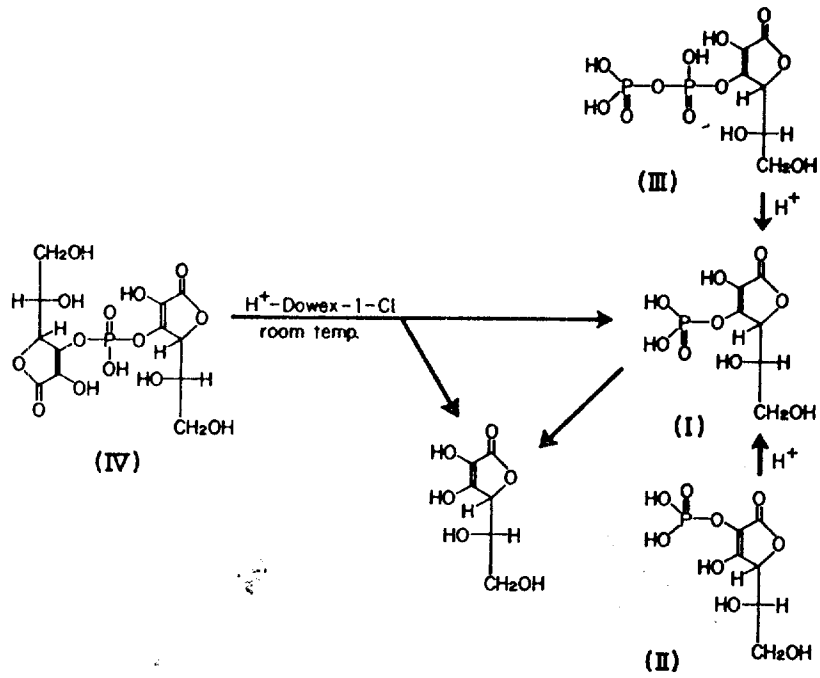

Chart 3.

observed on paper partition chromatography. A satisfactory separation and isolation of a group of these phosphates have been accomplished by ion exchange chromatography using Dowex-1-bicarbonate and a solution of sodium bicarbonate.[35]

An intense red coloration with ferric chloride and a lack of the iodine reducing power of each compound indicate the phosphoryl group being introduced into the hydroxyl group either on C_2 or C_3 of L-ascorbic acid. Elementary analysis and potentiometric titration showed the structure of each compound to be two isomeric ascorbic acid monophosphates, the pyrophosphate, and bis-ascorbic acid phosphate. On the ferric chloride test, which is specific for the enediol monophosphate,[39] three gave colors with the absorption maximum at 480 mμ, whereas one showed at 460 mμ. This suggests that the latter would likely be different from the others in the location attached to the phosphate.

Furthermore, the degradation reactions as shown in Chart 1 demonstrated that I and II are L-ascorbic acid 3- and 2-phosphate, respectively. NMR analysis also supported this conclusion. On mild acid hydrolysis or alkaline treatment the pyrophosphate, III was partially hydrolyzed with the almost quantitative liberation of one mole each of inorganic phosphate and I. This result indicates that the pyrophosphate group is attached to the 3-position of ascorbic acid. The location of the phosphoryl group in IV was confirmed on the basis of the NMR analysis and the hydrolysis reaction. Careful inspection of the NMR pattern showed that the two ascorbic acid moieties in IV were definitely equivalent and that the phosphoryl group was attached to the C_3 hydroxyl group of ascorbic acid, since only one set of the AB_2X pattern was existent, and the signals due to the C_4 proton appeared as a quartet by the couplings to both the phosphorus and the C_5 proton. Thus, IV was defined to be bis L-ascorbic acid 3,3′-phosphate. From these findings the phosphorylation reaction described above may progress as in Chart 2.[35]

Finally, Nomura, et al.[36] discovered a method for the preparation of L-ascorbic acid 3-phosphate by the selective phosphorylation of 5,6-isopropylidene-L-ascorbic acid. The reaction was achieved by the use of phosphorus oxychloride and a solvent of high polarity, e.g., water.

Chemical conversion of ascorbic acid phosphates

It has been shown that organic phosphates which possess neighboring hydroxyl groups are readily isomerized in acid solution.[37, 38] The migration of the phosphoryl group tends to occur in a direction away from the carbonyl group.[37] The observation that L-ascorbic acid 2-phosphate, upon acid treatment, isomerizes to the 3-phosphate in no way alters the generalization. The phosphoryl migration and the related partial hydrolysis are summarized in Chart 3.[35]

Physicochemical properties of ascorbic acid 3-phosphate

The major properties of the salts of ascorbic acid 3-phosphate which can be isolated as magnesium, calcium or sodium salt are described below:

Mg-Salt

Colorless, crystalline powder	
Melting point	Above 300° C
Elementary analysis	$C_6H_6O_9PMg_{3/2} \cdot 5H_2O$
Calculated	C=19.00; H=4.22; P=8.18; Mg=9.63
Observed	C=18.88; H=4.15; P=8.14; Mg=9.81
Specific rotation	$[\alpha]_D^{20} = +43.6°$ (C=1.6)
UV absorbancy	$\lambda_{max}^{0.1N\ HCl} = 237$ mμ, $= 0.98 \times 10^4$
	$\lambda_{max}^{0.1N\ NaOH} = 261$ mμ, $= 1.61 \times 10^4$
Infrared absorbancy	ν_{max}^{KBr} (cm⁻¹) 3400 (OH, broad), 1726 (C=O) 1605 (C=C), 1410, 1155, 1119
NMR (60 MC, D_2O):	3.85 (2H, J_1 = 6.64 cps); 4.16 (1H, J_1 = 6.64, J_2 = 1.6 cps); 4.61 (1H, J_2 = 1.6, $^4J_{HP}$ = 0.75 cps)
Dissociation constant (28.5° C, by spectrophotometry)	$pK_1 = 0.01$; $pK_2 = 3.27$; $pK_3 = 6.70$
Absorbancy of Fe^{+++} chelate in visible range	480 mμ (0.1N HCl)
Paper chromatography	Propanol:water:trichloroacetic acid (15:4:1) R_f = 0.35 (20° C, 24 hours)
Solubility	Readily soluble in water, but insoluble in organic solvents

Ca-Salt

Colorless, crystalline powder	
Melting point	Above 300° C
Elementary analysis	$C_6H_6O_9PCa_{3/2} \cdot 7H_2O$
Calculated	C = 20.64; H = 2.86; P = 8.87
Observed	C = 20.69; H = 3.12; P = 8.83
Solubility	Sparingly soluble in water, but insoluble in organic solvents

Na-Salt

Colorless, hygroscopic powder	
Elementary analysis	$C_6H_6O_9PNa_3 \cdot 1/2H_2O$
Calculated	C = 21.7; H = 2.11; P = 9.37
Observed	C = 21.5; H = 2.64; P = 9.77
Solubility	Readily soluble in water, but insoluble in organic solvents

L-Ascorbic acid 3-phosphate is stable against hydrolysis in neutral or an alkaline solution, but in an acid solution it is readily hydrolyzed to L-ascorbic acid and inorganic phosphate. In each instance apparent first order kinetics with respect to the phosphate were obtained by determining the remaining phosphate as shown in Figure 1. The pH-rate profile for the hydrolysis of L-ascorbic acid 3-phosphate over the range of pH 1-10 is shown in Figure 2 and, in acid below pH 1, the rate increases linearly with the acid concentration.[39] L-Ascorbic acid 3-phosphate, which is stable to iodine solution oxidation and to hydrolysis, showed much more stability on long storage as compared to that of L-ascorbic acid.

Mima and Matsuzaki[40] discovered boric acid and

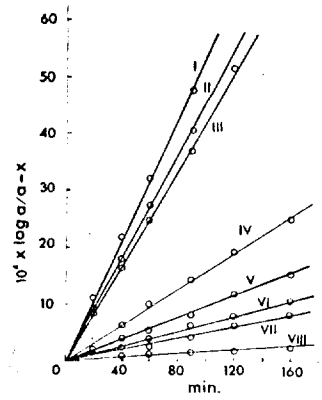

Fig. 1. Hydrolysis of L-AsA-3-phosphate at 100° at various pH values: I, 1.85; II, 2.90; III, 3.17; IV, 4.38; V, 4.96; VI, 5.73; VII, 6.73, VIII, 8.63.

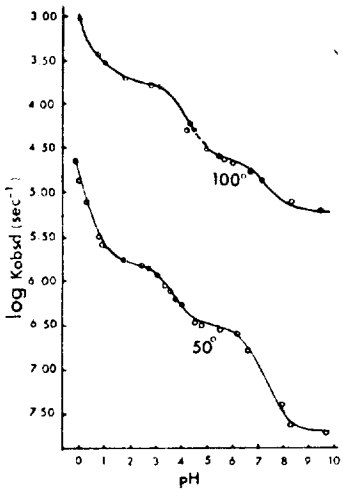

Fig. 2. Rates of hydrolysis of L-AsA-3-phosphate at 50° and 100°.

its salts to exert a satisfactory effect on stabilization of neutral and alkaline solutions of ascorbic acid phosphate (Table I).

The stability of ascorbic acid derivatives which are added to cosmetic creams (3%) is shown in Table II. Test ointments were filled in a tube, stored at room temperature and 40° C for one year, and changes in appearance were examined comparatively.

Table I. Stability of 1% L-AsA-3-phosphate solution at 40° C.

Buffer	pH	14 days	21 d.	28 d.
0.05 M-borax-0.1 N HCl	7.9	96.0%	95.5	95.1
0.05 M-borax	8.25	95.5	93.9	93.3
0.066 M-phosphate	7.9	85.2	84.2	83.9
Aqueous solution		90.8	89.1	88.5

Table II. Discoloration of cream containing AsA derivatives (3%) during storage.

Derivatives of AsA	1 week RT	1 week 40°	1 month RT	1 month 40°	2 mos. RT	2 mos. 40°	3 mos. RT	3 mos. 40°	1 yr. RT
AsA-phosphate Mg	-	-	-	-	-	-	-	-	±
AsA-3-stearate	-	-	±	+	±	+			
AsA-6-stearate	-	-	+	++	+	++	+	++	
AsA-3-benzoate	-	-	±	+	±	+	±	+	
AsA-3-benzoate-6-stearate	-	-	±	±	±	±	+	+	
AsA	±	+	+	++	++	+++			

- = no discoloration; ± = slightly yellowish-brown
+ = yellowish-brown; ++ = pronounced yellow brown
+++ = dark brown

Conversion of ascorbic acid phosphate to ascorbic acid in vivo

The conversion of the magnesium salt of ascorbic acid phosphate to ascorbic acid was examined by mixing the compounds with homogenates of the upper half of the small intestine, liver and an extract of skin.[25] In Figures 3 and 4 are shown the results of quantitative assay using 2,4-dinitrophenylhydrazine for free ascorbic acid so liberated. The conversion took place at a higher rate in neutral or acidic cultures than in alkaline solutions. The compound was incubated in N/20 HCl solution or pancreas fluid at 37°C for one hour for control purposes, but only 5% was hydrolyzed in the former and none in the latter. In their experiment on skin extracts, Takenouchi, et al.[11] proved that 90% of the magnesium salt of ascorbic acid phosphate was converted to ascorbic acid in three hours of incubation. In a similar experiment on ascorbic acid-2,6-dipalmitate it was revealed that the compound was hydrolyzed only at ⅓ the rate for the magnesium salt of ascorbic acid phosphate.

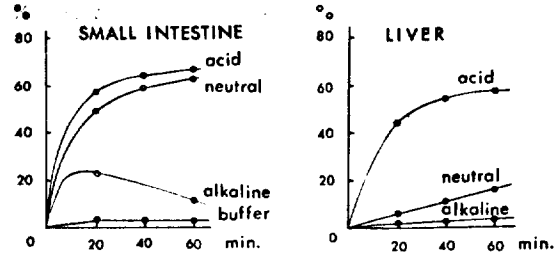

Fig. 3. Hydrolysis of Mg L-AsA phosphate in the homogenates of small intestine and liver. 37° C, 3.70 mg/g tissue/10 ml, buffer solution; 0.05 M, pH 4.8; acetate, 7.4; Krebs-Ringer phosphate, 10.0; carbonate-bicarbonate buffer.

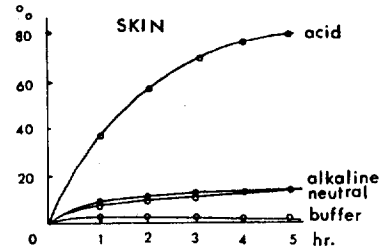

Fig. 4. Hydrolysis of Mg L-AsA phosphate in the crude extract of skin of guinea pigs. 37° C, 9.23 mg/2 g tissue/15 ml, pH pH 4.7; acetate, 7.2 and 9.4; veronal buffer.

A paper has been published on the ascorbic acid level in the skin of guinea pigs when a 3% magnesium ascorbic acid phosphate ointment is percutaneously administered.[42] That is, the hair of the back of adult guinea pigs was depilated over an area of 5 x 10 cm²; 5 grams of the test ointment were applied three days later; 1 gram of the treated skin area was collected at given intervals; the test ointment remaining on the skin surface was removed completely; and the ascorbic acid present in the skin sample was quantitatively assayed. The results are shown in Table III. The ascorbic acid level reached a level about 7 times the normal in 30 minutes; the skin sample was free of stratum corneum, however.

Table III. Contents of AsA in the skin of guinea pigs.

	AsA conc. (γ/g)
Control	2.0
30 min. after administration	15.0
60 " " "	10.1
120 " " "	7.5
180 " " "	5.2
240 " " "	11.3
300 " " "	7.5

These experiments revealed that ascorbic acid phosphate would be converted to ascorbic acid in the living tissues. What is the mechanism of this conversion? Hydrolysis by acid phosphatase may be the first consideration. Miura[43] reported the localized presence and distribution of acid phosphatase in fresh human skin. We, too, collected samples of human skin by a stripping method and detected acid phosphatase activity in the cells of the stratum corneum. These findings support the assumption that ascorbic acid phosphate will readily liberate ascorbic acid in the superficial layer of skin, and it is not necessary for the compound to reach the middle and deep layers.

The variations in the blood ascorbic acid level upon oral administration of magnesium ascorbic acid phosphate have been reported.[28] The compound was dissolved in 1 ml of water, and ascorbic acid also was dissolved in 1 ml of water. The solutions were orally administered to male guinea pigs in a dose of 54 mg of the test compound per animal

and 25 mg of ascorbic acid per animal; the blood ascorbic acid level was determined in the respective animals 1, 4 and 8 hours later. The results are shown in Figure 5. The blood level of both substances reached a peak in one hour and gradually fell thereafter. Magnesium ascorbic acid phosphate raised the blood ascorbic acid level in a similar way to ascorbic acid.

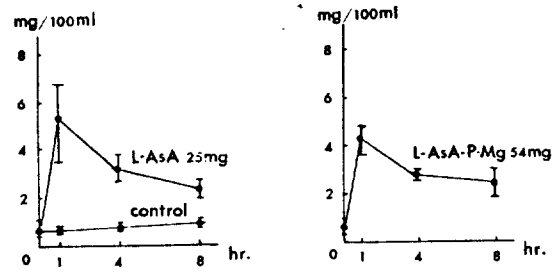

Fig. 5. Blood AsA level after oral administration of Mg L-AsA phosphate. Five guinea pigs, fasted for 24 hours, mean ± standard error.

Biological activity of ascorbic acid phosphate

As is evident from its chemical properties, ascorbic acid phosphate itself has no reducing action and may be considered to be free of biological activity. As observed in the conversion of this compound *in vivo*, it shows an antiscorbutic activity and pigmentation-suppressing activity through liberation of ascorbic acid.

The antiscorbutic activity of ascorbic acid phosphate and that of ascorbic acid by the oral route of administration were comparatively examined (Table IV).[28] When 1 mg of ascorbic acid per animal per day and an equal mole of magnesium ascorbic acid phosphate were administered, i.e., 2.1 mg/animal/day, both gave rise to a marked antiscorbutic effect but the activity of magnesium ascorbic acid phosphate was shown to be a little less than that of ascorbic acid.

Table V shows the results of percutaneous administration.[28] An antiscorbutic effect could be observed with 15.0 mg of magnesium ascorbic acid phosphate per animal per day and 7 mg of ascorbic acid per animal per day respectively, for 10 days. The effect of the former was slightly weaker than that of the latter, but the former showed a marked effect such as increase in the ascorbic acid level in the liver.

The comparative vitamin C activity of ascorbic acid derivatives is shown in Table VI.[44] The respective test compounds were orally administered to adult guinea pigs and the vitamin C activity calculated from the bleeding-preventive activity, alkali phosphatase activity and variations in body weight. Since the ascorbic acid derivatives have a greater molecular weight than ascorbic acid itself, these are shown in Table VI in the amount equivalent to free ascorbic acid. Index B stands for the vitamin C activity, with the activity of ascorbic acid as 100.

Table IV. Antiscorbutic activity of oral Mg L-AsA phosphate.

Group	No. of guinea pigs	Initial body weight(g)	Body weight gain(g)	Sub-cutaneous hemorrhage	Plasma alkaline phosphatase
Normal[1]	21	273	70 ± 4[2]	0/21	22.1 ± 1.4[3]
Deficient	22	288	-72 ± 7	21/22	3.5 ± 0.6
L-AsA					
0.5 mg/day	9	274	23 ± 14	3/9	17.1 ± 2.3
1.0 "	13	278	35 ± 5	1/13	17.1 ± 1.9
2.5 "	13	292	33 ± 5	0/13	18.8 ± 1.8
5.0 "	15	276	34 ± 7	0/15	22.8 ± 2.6
L-AsA phosphate-Mg					
1.05 mg/day[4]	9	314	-8 ± 13	3/9	10.4 ± 0.5
2.1 "	7	302	9 ± 9	1/7	15.7 ± 1.1
5.25 "	4	308	39 ± 6	0/4	20.1 ± 1.2
10.5 "	4	284	21 ± 7	0/4	23.0 ± 2.5

[1] = Oriental GC-5 [2] = during the 11th-21st day [3] = King Armstrong unit
[4] = Equimole to 0.5 mg of L-AsA Mean ± standard error

Table V. Antiscorbutic activity of percutaneous Mg L-AsA phosphate (in cream).

Group	No. of guinea pigs	Initial body weight(g)	Body weight gain(g)	Sub-cutaneous hemorrhage	Plasma alkaline phosphatase	Liver weight (g/100 g b. wt.)	Liver AsA content (mg %)
Normal	4	230	55 ± 5	0/4	24.1 ± 0.4	3.83	30.8 ± 1.6
Deficient	10	249	-40 ±14	10/10	4.0 ± 0.7	4.02	0.5 ± 0.1
VC-cream[1]							
L-AsA 3.5 mg/day	4	255	-4 ±10	2/4	14.4 ± 1.3	4.35	2.0 ± 0.5
7.0 "	3	262	7 ±28	0/3	17.1 ± 2.4	4.37	5.8 ± 2.2
14.0 "	2	263	56	0/2	22.8	3.96	14.6
VC-P-cream[1]							
L-AsA phosphate-MG							
7.5 mg/day	3	263	-35 ±20	3/3	7.7 ± 1.1	3.92	0.4 ± 0.1
15.0 "	5	260	20 ± 7	1/5[2]	16.8 ± 3.9	3.73	3.3 ± 0.3
30.0 "	2	255	56	0/2[2]	24.4	4.03	10.2

[1] = 1.4% VC-cream, 3% VC-P-cream
[2] = 1/5 (15 mg/day) and 0/5 (30 mg/day) were observed in the other experiment (omitted)

Table VI. VC activity of AsA derivatives.

Group	Dose (mg/day)	Anti-hemorrhage(%)	Recovery[1] of phosphatase (%)	Recovery of body weight (%)	Index B (%)
Normal		100	100	100	
Deficient control		0	0	0	
L-AsA	5.0	98	107	65	100.0
Ascorbigen	8.95[2]	6	43	15	23.0
AsA-2,5,6 tribenzoate	14.35	50	37	36	45.0
AsA-2,6 dibenzoate	11.30	45	38	64	53.8
AsA-6-benzoate	8.25	100	103	78	104.0
AsA-3-stearate	13.00	82	68	67	73.5
AsA-6-stearate	13.00	100	130	84	115.0
AsA-Phosphate Ca	11.30	90	73	64	83.5
AsA-Phosphate Mg	10.90	100	96	75	100.0

[1] = Ph-tase - activity of serium alkali phosphatase
[2] = equimolar to 5 mg of L-AsA

These tables indicate that:

1) even the compounds with substitution at the 3rd position of ascorbic acid show a vitamin C activity upon oral administration;

2) there are no derivatives of ascorbic acid which show a higher vitamin C activity than ascorbic acid itself, even the most potent one being no better than a ratio of 1:1;

3) the esters of ascorbic acid have a lower vitamin C activity as a larger number of radicals are substituted; and

4) a compound in which a higher fatty acid is substituted in the 3 position is lower in vitamin C activity than a compound which is similarly substituted at the 6 position containing a free enediol.

Toxicity and side effects of magnesium ascorbic acid phosphate

The acute toxicity of magnesium ascorbic acid phosphate is shown in Table VII.[45]

Table VII. LD_{50} of Mg AsA phosphate (mg/kg).

Animal	Test material	P.O.	I.P.
Rat	AsA phosphate Mg	10,000	5,187
	AsA	10,000	2,508
Mouse	AsA phosphate Mg	10,000	2,434
	AsA	10,000	3,240

The chronic toxicity was determined in rats. Doses of 0.5, 1.0, and 2.0 grams of test compound/kg/day and 1.0 gram of ascorbic acid/kg/day were orally administered for 6 months, but no abnormalities were found in the body weight, blood picture, appetite, and weight and histological findings of the organs.[45]

The toxicity to fetuses was tested in mice and rats, and magnesium ascorbic acid phosphate was proved to be free of any teratogenic effect.[46]

No abnormalities were found when the irritant effect of 3% magnesium ascorbic acid phosphate ointment was tested by applying it to the skin of the external ear of both humans and rabbits for one month. The histological examination of the external ear of rabbits further revealed no anomalies but rather showed the ointment base to have caused acanthosis in some animals.[47]

Percutaneous absorption of magnesium ascorbic acid phosphate

It can be readily assumed that ascorbic acid phosphate, when absorbed through the skin, would be hydrolyzed to ascorbic acid. Imai, et al.[28] applied a 3% magnesium ascorbic acid phosphate cream to the depilated area of the back of guinea pigs, accelerated absorption by ODT with saran wrap, and visually observed the ascorbic acid that was liberated intracutaneously in terms of silver particles formed, using the $AgNO_3$ method. The result is shown in Figure 6. The silver particles were found intercellularly in the epidermis 24 hours after application, but were retained near the basal cell layer thereafter.

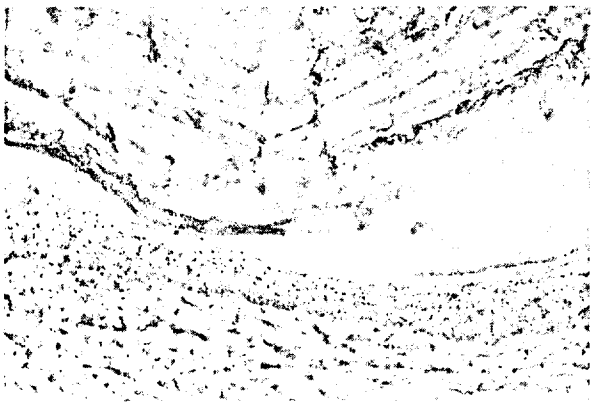

Fig. 6. Distribution of AsA in skin of guinea pigs applied with Mg AsA phosphate cream. AsA was visualized as Ag particles.

An experiment was carried out by Takenouchi, et al. in which a 3% magnesium ascorbic acid phosphate cream was applied to an area of the skin of guinea pigs from which the stratum corneum and hair had been removed together by a depilatory cream containing thioglycolic acid.[42] In this case considerably marked absorption took place in 20 minutes and the compound penetrated to the lower layer of epidermis in one hour.

The above cream was applied to both lower limbs of 3 healthy human adults; urine samples were collected up to 8 hours later; and the urinary output of ascorbic acid was determined. The results are shown in Table VIII.[41]

Table VIII. Urinary VC after application of Mg AsA phosphate cream and control (man).

	Control	AsA phosphate cream
Before application	34.5 μg/10 ml	28.2 μg/10 ml
After 2 hours	37.3	59.5
After 4 hours	37.8	91.5
After 6 hours	32.1	62.1
After 8 hours	27.4	57.7

The antiscorbutic activity and increase in the ascorbic acid level in the skin and liver on percutaneous administration of magnesium ascorbic acid phosphate support the percutaneous absorption of this compound.

Effect of ascorbic acid derivatives on melanogenesis

Suppression of the action of tyrosinase, collected from potatoes, by ascorbic acid and magnesium ascorbic acid-3-phosphate was tested using dl-DOPA as substrate.[48] It was found that the action of tyrosinase was considerably inhibited by a concentration of 2×10^{-4} M of ascorbic acid, but scarcely suppressed by even 1×10^{-3} M of magnesium ascorbic acid-3-phosphate (Table IX). Dephosphorylation is thus essential for inhibition of melanogenesis by ascorbic acid phosphate.

Table IX. Inhibitory action on DOPA-chrome formation.

Sample		Inhibition %
L-AsA	5×10^{-5} M	18.3
	1×10^{-4}	36.0
	2×10^{-4}	69.0
	4×10^{-4}	98.6
L-AsA-3-P-Mg	5×10^{-5} M	-1.4
	1×10^{-4}	0.7
	2×10^{-4}	2.1
	4×10^{-4}	0.7
	1×10^{-3}	2.1

dl-DOPA = 100 γ/3.2 ml of 0.1 M phosphate buffer (pH 6.9)
Enzyme solutn. = 0.2 ml
All reactions were run at 25° C

Application of ascorbic acid phosphate in dermatology

A series of experiments were carried out by Takenouchi, et al.[41] on the topical application of an ointment containing magnesium ascorbic acid phos-

phate; it was proved that the compound exerts a pigmentation-suppressing effect.

1) *Erythema-inhibitory effect.* An ointment containing magnesium ascorbic acid phosphate was applied to the upper arm of human subjects; ODT with saran wrap was performed; the test sample was adequately removed 4 hours later; and the treated area was irradiated with quartz lamp for different periods, viz., 5, 10, 15, 20, 25, and 30 seconds. The right upper arm was not treated with the ointment. Marked erythema developed on the following day on the right upper arm which had been irradiated for 10 seconds, but erythema was only slightly noticeable on the ointment-treated area of the left upper arm which had been irradiated for 15 seconds. The pretreatment with the ointment containing magnesium ascorbic acid phosphate thus showed an inhibitory effect against erythema by ultraviolet irradiation.

2) *Artificial pigmentation.* Pigmentation was induced beforehand on the skin of the flexor side of both forearms of human subjects by irradiation with UV ray of a long wavelength; an ointment containing magnesium ascorbic acid phosphate was applied to the left arm once a day, and the right arm was left untreated. This schedule was continued for 80 days. The variations in pigmentation are shown in Figure 7. The darkness of skin color was expressed in terms of lightness. The area where the test ointment was applied was 2-3% lower in pigmentation.

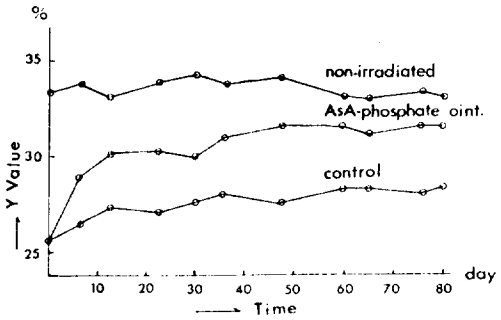

Fig. 7. Effect of Mg AsA phosphate ointment on UV ray erythema.

On the other hand, when compared with the results of similar experiments on the oral administration of 1,000-2,000 mg of ascorbic acid daily the application of an ointment containing magnesium ascorbic acid phosphate was found to be more effective. From the percutaneous absorption of ascorbic acid phosphate, and also from the conversion of this compound to ascorbic acid and retention as such, it may be considered that the ascorbic acid level at the site of application was raised, and the application thus was effective for suppressing pigmentation.

3) *Other pigmentations.* Chloasma, Riehl's melanosis, freckles, pigmented naevus, and drug rash are problems that are regarded as intractable. The application of potent agents may be considered for these types of pigmentation, but these agents are usually accompanied by side effects such as irritation. The prolonged, large-dose oral therapy with vitamin C is now accepted as the safest and most effective means. A paper has been published on the

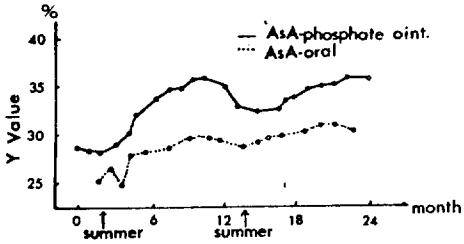

Fig. 8. Effect of Mg AsA phosphate on chloasma.

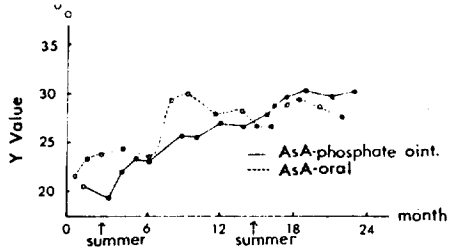

Fig. 9. Effect of Mg AsA phosphate on Riehl's melanosis.

clinical application of ascorbic acid phosphate ointment for such purposes.[49]

A 3% magnesium ascorbic acid phosphate ointment was applied to 18 patients with chloasma for a long period, and 1,000-2,000 mg of ascorbic acid daily were orally administered to another 22 patients for comparison of the efficacy. The results are shown in Figure 8. The effects were assessed in terms of lightness by the use of a reflective photometer.

Lightness gradually rose, showing a transient fall in summer and a rise in winter. In other words, pigmentous spots became less dark while showing seasonal variations in darkness. To cite the lightness of the flexor side of the forearm of the Japanese measured by the reflective photometer for reference, it is 50.7% in congenital albinism, 34.9% in normal women, and 30.9% in normal men, and the difference in lightness between the pigmented spot and adjacent healthy part was 5% in cases where the pigmentation was recognized as morbid. As shown in Figure 8, a 7% fall in lightness by the application of ascorbic acid phosphate ointment and a 5% fall by oral administration of ascorbic acid may be considered to represent a restoration of skin color to normal.

A comparative study was undertaken by topical application of a 3% magnesium ascorbic acid phosphate cream to 9 patients with Riehl's melanosis and by oral administration of ascorbic acid to 8 patients with the same disturbance.[49] The results are shown in Figure 9. As observed in the chloasma cases, lightness was improved in about 30% in both groups 25 months later. The oral administration was more effective in the first year of treatment, but the topical application was more effective in the second year. The finding that both topical and oral administration were less effective for Riehl's melanosis than for chloasma may be considered to originate in the fact that chloasma is of epidermic nature, viz., these chiefly come from the pigmentation in the upper layers than from the basal cell layer, whereas proliferation of pigment in the upper layer of the corium is the main cause of Riehl's melanosis, and

it is difficult for the active substance to reach the deep part of the skin even when it is absorbed.

The treatment may be considered to be effective in cases of skin eruptions, post-traumatic pigmentation, and senile pigmentation where the pigmentation is relatively superficial and is brown-black in color.

Summary

Notwithstanding the fact that ascorbic acid is effective and safe for the treatment of skin pigmentation, it is less stable as an ingredient of cosmetics. Recently a number of ascorbic acid derivatives which are antiscorbutically active and enhanced in periodical stability have been introduced, and among them ascorbic acid-3-phosphate has been shown to be satisfactorily stable in application for cosmetics and to be dephosphorylated into the parent ascorbic acid by phosphatase in the living cell. Actually, it exhibited not only an antiscorbutic effect but also a bleaching effect on pigmentation. The correlation of skin pigments with the phosphate ester of ascorbic acid was taken up here from the chemical and biological aspects.

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ON THE METABOLITES OF ASCORBIC ACID, ESPECIALLY OXALIC ACID, ELIMINATED IN URINE, FOLLOWING THE ADMINISTRATION OF LARGE AMOUNTS OF ASCORBIC ACID[1]

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Oxalic acid has been thought to be one of the metabolites of AsA. Burns *et al.* (1—3) have found the presence of a considerable amount of radioactive oxalic acid in urine following the administration of AsA-C^{14}. After carrying out the similar experiments Lamden *et al.* (4) have reported that, though the increase of oxalic acid eliminated in urine was not appreciable following the oral administration of less than 3 g of AsA per day, the urinary excretion of oxalic acid markedly increased after oral administration of 9 g per day. In view of the observations made by Hayaishi *et al.* (5), oxalic acid does not seem to be necessarily the end product of carbohydrate metabolism, but is utilized as a source of energy or carbon. It has not been elucidated, however, how the large amount of oxalic acid present in nature is utilized. The important aspect of the problem concerning this substance seems to be the excretion of calcium as calcium oxalate leading to the formation of urinary calculi when the substance is produced in large amounts in the body. Large amounts of AsA have in recent years been used for the treatment of pigment anomaly in the field of dermatology. The results of this treatment, as determined with lustermeter, show that there was an increase in the lightness of color of the skin one year after the treatment (6). In this treatment, large amounts of AsA was given to patients over a long period of time, so that the formation of oxalic acid and its influence would warrant further clinical as well as experimental studies.

We have carried out the studies on the metabolites of AsA, especially oxalic acid, which are produced following the administration of massive doses of the vitamin. The influence of oxalic acid on the formation of oxalate calculi in rats has also been a subject in the present studies.

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[1] Vitamin C in Relation to Pigment Anomaly. II. Following abbreviations were used: AsA, ascorbic acid; DAsA, dehydroascorbic acid; DKG, 2,3-diketogulonic acid.

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EXPERIMENTAL

1. *Experiments in Human Subjects*

AsA was given orally or intravenously to healthy, normal subjects in a dose of 3 g per day, and AsA and DAsA as well as DKG were determined in 24 and 48-hour urines by the 2,4-dinitrophenylhydrazine method of Roe modified by Teruuchi (7). At the same time, the oxalic acid in the same urine was determined by the method of Powers and Levatin (8). Urine was collected in a urinal containing 20% metaphosphoric acid and 3.3% thiourea according to Lamden (4) to prevent the oxidation and decomposition of AsA. This stabilizing agent was shown by the experiments of the authors to be satisfactory. Detemination of the urinary metabolites was also carried out after 3 and 9 g of AsA, respectively, were orally administered every day for consecutive two weeks.

2. *Experiments in Animals*

AsA-C^{14}, 50 μc/0.8 mg, was used in this experiment. The distribution in the body of radioactive AsA as well as in urine was measured in rats, guinea pigs and man (prickle cell cancer). 50 mg and 1 g of non-radioactive AsA were used as carriers for 1 mg of AsA-C^{14} in animals and man, respectively. Food and waterwere supplied *ad libitum*. The $C^{14}O_2$ excreted in expired air was determined according to Mackenzie (9). The expired air was passed through a solution of 2.5 *N* NaOH, to which $BaCl_2$ was added. An aliquot of the sedimented $BaCO_3$ was measured for C^{14} with a gas flow counter with correction in accordance with the table of Yankwitch (10).

Determination of C^{14} in urine and feces as well as body organs was carried out as follows. About 10 mg of $BaSO_4$ was added to a given amount of AsA-C^{14}, and the radioactivity was measured in counts per minute and corrected in accordance with the table of Yankwitch. The radioactivity of C^{14} administered was subtracted from the corrected total counts to obtain the values in excretions and organs.

A suitable amount of $BaSO_4$ was added to 0.2 ml of urine, to an aliquot of the extract of the organ, prepared similarly as described in the assay of total AsA, or to an aliquot of the aqueous extract of feces so as to give about 10 mg. It was placed on the Petri dish, dried under infrared irradiation and the radioactivity was measured. Correction was made in accordance with the table of Yankowitch after weighing the Petri dishes.

Radiactive oxalic acid was determined by placing on the Petri dish the calcium oxalate obtained from urine according to Powers and Levatin (4), drying and weighing. Correction of the counts was made in accordance with the table of Yankowitch.

3. *Effects of the Administration of Massive Doses of AsA on the Formation of Urinary Calculi*

For studying the effects of the massive dose of oxalic acid on the formation of urinary calculi, following experiments were performed. A lead ball weighing about 50 mg was inserted as a nucleus into each bladder of male albino rats, about 100 g in weight. AsA was intramuscularly injected for consective 17 days, at the end of which the animals were bled to death and the nucleus and the

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salts attached to it were withdrawn. The salts were dried and weighed. The calculi were dissolved in 50 ml of 5% HCl. The solution was boiled for 10 to 15 minutes on a water bath. An aliquot of the solution was analyzed for oxalic acid. Attempts were also made to test the solution for urate, ammonium salts, cystine phosphate and magnesium.

RESULTS

1. Metabolites of AsA Eliminated in Urine Following Administration of Large Amounts of AsA

Oral Administration of 3 g of AsA.—Three subjects were given 3 g of AsA per day (in 3 installments), and the metabolites eliminated in urine were measured before and after administration. The amount of total AsA (average of three subjects) was 13 mg, of which 57% was AsA; 42% DAsA; and 0% DKG, and that of oxalic acid was 18.64 mg. The values 24 and 48 hours after administration of AsA were 446 and 430 mg total AsA, of which 79 and 67% were AsA; 16.7 and 27.4% DAsA; and 4.1 and 5.4% DKG; and 29.4 and 25.27 mg oxalic acid, respectively (Table 1).

TABLE 1

AsA and Its Metabolites Eliminated in Urine Following Oral Administration of 3 g of AsA in Man

Time after administration	Case	Total AsA	AsA	DAsA	DKG	Oxalic acid
		mg	*mg*	*mg*	*mg*	*mg*
Before	1	6.20	0	6.20	0	18.72
	2	21.37	16.62	4.75	0	11.40
	3	12.00	6.60	6.00	0	25.80
	Average (%)	**100**	**57.2**	**42.8**	**0**	**18.64**
24 hr	1	326.4	291.4	27.8	7.2	26.69
	2	365.0	232.5	87.5	45.0	17.30
	3	647.4	534.9	107.66	4.39	44.32
	Average (%)	**100**	**79.2**	**16.7**	**4.1**	**29.43**
48 hr	1	275.0	133.9	120.5	23.7	20.35
	2	418.0	323.4	54.5	10.2	17.82
	3	597.6	410.4	180.6	6.6	37.65
	Average (%)	**100**	**67.2**	**27.4**	**5.4**	**25.27**

It will be seen that about 30% of AsA administered was eliminated in urine in 48 hours, whereby DKG produced by the degradation of AsA in the body increased with time. However, the increase of oxalic acid was not marked. Shimazono *et al.* (11) have shown that DAsA formed by oxidation of AsA is decomposed to DKG, which, in turn, is oxidized to produce oxalic acid and L-threonic acid and at the same time DKG is partially decarboxylated to produce L-xylonic acid and L-lyxonic acid. The results given above seem to show, however, that the oxidation of DKG to produce oxalic acid was not so marked as has been pointed out.

Intravenous Injection of 3 g of AsA.—The amount of total AsA eliminated in urine before administration of AsA (average of three subjects) was 63 mg,

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of which 82.5% was AsA; 14.8% DAsA; and 2.7% DKG, and that of oxalic acid was 34.4 mg. Following the administration of 3 g of AsA per day (given in three installments), the corresponding figures were 457 mg for total AsA, of which 72% was AsA; 17.9% DAsA; and 10.1% DKG, and 26.6 mg oxalic acid after 24 hours; and 79 mg for total AsA of which 84.2% was AsA; 13.7% DAsA; and 2.1% DKG, and 35.6 mg oxalic acid after 48 hours. It will be seen that the increase in the amount of oxalic acid was not marked after intravenous injection as after oral administration. However, the amount of DKG, one of the metabolites of AsA, showed an increase in proportion to the amount of AsA administered (Table 2).

TABLE 2

AsA and Its Metabolites Eliminated in Urine Following Intravenous Administration of 3 g of AsA in Man

Time after administration	Case	Total AsA	AsA	DAsA	DKG	Oxalic acid
		mg	*mg*	*mg*	*mg*	*mg*
Before	1	103.5	92.0	10.05	1.45	63.94
	2	59.2	47.2	10.4	1.60	28.48
	3	25.25	15.87	7.5	1.87	10.72
	Average (%)	**100**	**82.5**	**14.8**	**2.7**	**34.38**
24 hr	1	782.0	542.0	160.0	80.00	58.60
	2	450.0	336.0	65.82	48.20	20.60
	3	140.0	110.0	0.01	10.00	10.56
	Average (%)	**100**	**72**	**17.0**	**10.10**	**26.58**
48 hr	1	138.0	114.0	21.6	2.40	68.4
	2	57.0	49.0	6.00	0.00	24.6
	3	44.0	36.5	4.84	2.64	13.90
	Average (%)	**100**	**84.2**	**13.7**	**2.10**	**35.63**

Oral Daily Administration of 3 g AsA for Two Weeks——AsA, 3 g, was administered orally to three subjects daily for two consecutive weeks. The metabolites were estimated in 24-hour urine collected on the last day of administration and the following valuse were obtained (averages of 3 subjects): total AsA, 476 mg, of which 90.8% was AsA; 5.9% DAsA; and 3.3% DKG, and oxalic acid, 30.97 mg (Table 3).

TABLE 3

The Metabolites of AsA Eliminated in Urine Following the Administration of 3 g of AsA per Day for a Long Period in Man

Case	Total AsA	AsA	DAsA	DKG	Oxalic acid
	mg	*mg*	*mg*	*mg*	*mg*
1	409.2	381.3	21.7	7.2	4.08
2	202.5	180.0	11.25	11.25	10.62
3	815.3	734.41	51.67	29.2	58.21
Average (%)	**100**	**90.8**	**5.9**	**3.32**	**30.97**

Oral Daily Administration of 9 g AsA for Three Consecutive Days——The amount of oxalic acid found in the 24-hour urine following the oral daily administration of 9 g AsA (given in three installments) for 3 consecutive days was 43.2 mg (Table 4).

TABLE 4

The Metabolites of AsA Eliminated in 24-hour Urine Following the Administration of Large Amounts of AsA (an average of 3 cases)

Mode of administration	Total AsA	AsA	DAsA	DKG	Oxalic acid
	mg	*mg*	*mg*	*mg*	*mg*
Before Ad.	62.6	51.7	9.3	1.61	18.6
3 g per day orally	146.2	352.9	71.3	18.8	29.4
3 g per day intravenously	157.3	329.3	81.9	46.9	26.6
2 g per day orally for consecutive 2 weeks	175.6	131.9	28.2	15.9	32.3
9 g per day orally for consecutive 3 days					43.2

In short, AsA was found to be the major portion of the metabolites of AsA eliminated in urine following the administration of a large amount (3 g) of the vitamin both orally and intravenously for 2 consecutive weeks, the urinary DKG was increased with the rise of the amount of AsA administered, and oxalic acid, produced by oxidation of DKG, not markdly increased in urine until 9 g AsA was given orally for 3 consecutive days.

2. *Administration of AsA-C^{14} to Man and Animals*

AsA-C^{14} was given orally to man (a patient with prickle cell cancer) and intraperitoneally to rats and a guinea pig, and the relation between oxalic acid and other metabolites of AsA in urine was investigated.

Recovery of Administered AsA-C^{14} — The principal purpose of this experiment was the determination of radioactive oxalic acid eliminated in urine following the administration of AsA-C^{14}. Therefore, the recovery of C^{14} eliminated in expired air, feces and urine must be complete, and the distribution of C^{14} in organs can be estimated accurately. As can be seen from Table 5, 86.8% of the C^{14} administered was recovered from the guinea pig, while 85.7 and 77.9% were recovered from rat No. 1 and rat No. 2 respectively. It would therefore be justified to discuss the amounts of radioactive oxalic acid in urine.

TABLE 5

C^{14} Excreted in Urine, Feces and Expired Air and in Organs Following the Administration of AsA-C^{14}

	Guinea pig	Rat No. 1	Rat No. 2	Man (prickle cell cancer)
24-hour urine	61.1	63.1	48.0	
48-hour urine	1.48		0.85	
Oxalic acid in 24 hours	(1.58)	(1.18)	(0.58)	0.16
in 48 hours	0.19		0.01	
$C^{14}O_2$ in expired air	5.0	3.49	1.2	
Feces in 24 hours	0.24	0.3	0.12	Expired air was examined for 6 hours
in 48 hours	0.19		0.08	
C^{14} in various organs	5.87	6.40	8.56	
Residual C^{14}	12.14	12.12	19.13	
Recovered C^{14}	86.8	85.70	77.99	

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C^{14} Eliminated in Feces and Urine: The C^{14} eliminated in feces and urine was in the range of 48 to 63% (urine) and 0.2 to 0.43% (feces) of the amount administered.

C^{14} Eliminated in Expired Air: In the expired air of the guinea pig, 5.5% (in 24 hours) of the C^{14} administered was detected, while 3.49 and 1.2% were found in the expired air of rat No. 1 and No. 2 respectively, a finding not in agreement with that reported by Burns *et al.* (1, 2). They found 40% of C^{14} in the expired air and 10 to 20% in urine. The discrepancy may be due to the experiments conducted in AsA-deficient guinea pigs receiving orally radioactive AsA. Our experiment showed that the $C^{14}O_2$ in the expired air following intraperitoneal injection of AsA-C^{14} was, in counts per minute, 5,250 after 1 hour, 23,327 after 2 hours, 49,722 after 3 hours, 114,897 after 4 hours, 45,259 after 4 to 7 hours and 338,784 after 4 to 24 hours hours, the total in 24 hours being 577,239 corresponding to 5.45% of the dose administered. Similar tendency was also observed in rats, though the values were lower than those in the guinea pig.

Incorporation of AsA-C^{14} in Organs: Table 5 shows the results of the experiment. It will be seen that the counts were greater in heavy organs as the liver. Burns assumes the C^{14} in organs not to be the metabolites but AsA *per se*. Incorporation of the labelled vitamin per g tissue was about the same in the liver, kidneys, muscles, skin and brain. However, the incorporation was markedly greater in the adrenals, lungs and bones, the counts being 6.27, 2.80 and 2.34 times as high as those in the liver. It is known that AsA plays an important role in the adrenals which are rich in the vitamin. Much AsA seems to be metabolized in the lungs serving as an excretory organ and in the bones for producing collagen. The ratio of the total AsA of each organ to radioactivity is given in Table 6. The values varied from organ to organ, 2 to 6%. The bones gave the highest value of 6.8%, followed by the adrenals and kidneys, 4.0%.

TABLE 6

C^{14} Found in Organs of Guinea Pig 48 Hours after Intraperitoneal Administration of 1 mg of AsA-C^{14} (amount of AsA given, 43.57 mg)

Organs	Wet weight	Radioactivity	Total AsA	Relative counts per g (counts found in liver taken as 1)	C^{14} found in organs
	g	*cpm*	*mg*	*per cent*	
Liver	6.9	38,318	3.85	1	4.07
Kidneys	2.0	12,160	2.20	1.09	2.27
Muscles	2.0	8,300	2.5	0.75	1.36
Lungs	1.3	20,280	3.6	2.80	2.3
Cerebrum	3.0	16,050	2.65	0.96	2.49
Bone femur	2.0	24,930	1.5	2.24	6.81
Adrenals	0.2	7,300	3.7	6.57	4.05
Skin brown	2.0	11,580	0.75	1.04	3.16
Skin dark	2.0	9,200	1.35	0.82	1.40
Skin white	2.0	13,160	0.75	1.18	2.0

Elimination of AsA Metabolites in Urine — AsA-C^{14} was administered orally and intraperitoneally to a guinea pig and rats, and AsA and its metabolites eliminated in urine were determined. The results are given in Table 7. The C^{14} found in the urine of the guinea pig was 61.4% in 24-hour urine and 1.48%

TABLE 7

C^{14} and the Metabolites of AsA Eliminated in Urine Following the Administration of AsA-C^{14} in a Guinea Pig, Rats and Man

Amounts given: guinea pig, 43.57; rat No. 1, 50.98; rat No. 2, 43.57; man, 1.873 mg.

		Relative C^{14}		Metabolities of AsA				
		Total AsA-C^{14}	Oxalic acid-C^{14}	Total AsA	AsA	DAsA	DKG	Oxalic acid
		per cent		*mg*	*mg*	*mg*	*mg*	*mg*
Guinea pig	in 24 hr	61.4	1.58	22.5	20.0	1.92	0.52	1.470
	in 48 hr	1.48	0.19	15.8	10.62	3.07	2.17	0.41
Rat No. 1	in 24 hr	63.1	1.18	29.25	26.70	1.58	0.975	0.917
No. 2	in 24 hr	48.0	0.53	31.05	23.25	5.35	2.47	1.089
	in 48 hr	0.85	0.04	12.76	8.58	2.20	1.198	0.847
Man			0.16					

in 48-hour urine of the C^{14} administered. In the case of rats 63.1 (No. 1) and 48% (No. 2) were found in the 24-hour urine and 0.85% (No. 2) in the 48 hour-urine. The amounts of the labeled oxalic acid in urine were 1.77% (0.771 mg) in the guinea pig, 1.18% (0.601 mg) in the rat No. 1, 0.57% (0.548 mg) in the rat No. 2, and 0.16% (2.996 mg) in man of the amount of AsA-C^{14} administered. On the other hand, the amounts of total urinary oxalic acid were 1.88 mg in the guinea pig (in the 24-and 48-hour urine), 0.917 mg in the rat No. 1 and 1.936 mg in the rat No. 2. Accordingly, the labeled oxalic acid derived from AsA was 41.0% in the guinea pig, 65.5% in the rat No. 1, 12.8% in the rat No. 2 and 10% in man of the total urinary oxalic acid. In the guinea pig, the total AsA in the 24-hour urine was 28.5 mg, of which 20 mg was AsA, 1.92 mg DAsA and 0.975 mg DKG. It corresponds to 56.4% of the amount administered. In the rat No 1, the total AsA in the 24-hour urine was 29.25 mg, of which 26.70 mg was AsA, 1.58 mg DAsA and 0.975 mg DKG. It corresponds to 57.3% of the amount administered. In the rat No. 2, the total AsA in 24-hour urine was 31.05 mg, which corresponds to 71.2% of the amount administered. These figures were in good agreement with the percentage excretions calculated from the radioactivity.

The radiochromatogram and actigram obtained with the alcohol extract of the lyophilized urine samples revealed the spots of AsA and DAsA at *Rf* 0.3 and 0.4, respectively, but no other radioactive substances were detected.

In short, after oral administration of AsA-C^{14} to man and its intraperitoneal administration to animals (fed on a normal diet) 20 to 60% of the oxalic acid derived from AsA was eliminated in 48-hour urine in the animals and less than 10% in man. As shown by our experiments in human subjects, a great dose of AsA did not result in the excretion of much oxalic acid. After intraperitoneal injection of 50 mg AsA-C^{14} in animals 1 to 1.5% of AsA administered was eliminated as oxalic acid in the urine. In man, however, after administering 1.8 g AsA, only 0.16% of it was eliminated as oxalic acid.

Effect of Oxalic Acid Derived from AsA on the Experimental Formation of Urinary Calculi in Rats — A lead nucleus was inserted into each urinary bladder of 15 rats, and 0.5 mg of AsA was injected intramuscularly to the animals

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every day for deposition of the oxalic acid derived from AsA on the nucleus. Five rats were used as the control. Many of them died of infection in the first experiment of 50 rats, which were excluded from the data. The weight of the nucleas rose in weight by 5.6 mg in the control, whereas the corresponding figure was 39.6 mg in the animals receiving AsA consecutively, showing apparently a marked calculus formation around the nucleus (Table 8).

TABLE 8

Formation of Calculi Around Lead Nuclei Following Intramuscular Injection of 5 mg AsA for 17 Days in Rats

Rat No.	Formation of calculi	Weight of calculi	Amount of oxalic acid
		mg	*mg*
	Control rats		
74	− to ±	3.5	0.433
75	− to ±	7.7	0.162
76	− to ±	4.7	0.248
77	− to ±	2.5	0.496
78	± to +	9.1	0.256
Average		**5.6**	**0.326**
	Experimental rats		
52	− to ±	39	0.962
56	++	117.7	0.430
58	++	48.9	0.024
59	+	13.8	0.318
60	− to ±	3.4	0.624
61	− to ±	5.2	1.050
62	++	29.9	0.736
63	+	24.5	0.268
64	− to ±	12.4	0.978
65	++	33.3	0.433
66	++	29.2	0.678
67	++	39.1	0.512
69	+	21.4	1.074
70	±	6.2	0.716
72	− to ±	7.1	1.716
Average		**39.6**	**0.733**

The amount of oxalic acid in the calculi formed around the nucleus was determined to be 0.326 mg in the control and 0.733 mg in the AsA group, possibly showing the increase in the weight of calculi to be due to the deposition of oxalic acid derived from AsA. Besides oxalic acid, phosphates, ammonium salts and urates were detected. Ammonium salts were slightly increased in AsA group as compared with the control. Scarcely any difference was found between the control and AsA group in the amounts of other salts.

DISCUSSION

Since a great dose of AsA is used in the field of dermatology, the effects of the vitamin on the formation of oxalic acid and of urinary calculi in man and animals were investigated. For this purpose, a lead nucleus was inserted into the urinary bladder of rats. AsA is given in large amounts to patients with pigment anomaly (chloasma, pigmentation following inflammation and Riel melanosis) for a prolonged

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period. As has already been reported by the authors, AsA has following actions: (*a*) inhibition of melanogenesis, (*b*) conversion of oxidized melanin into the reduced form, and (*c*) prevention of erythema caused by ultraviolet rays. Therefore, the use of AsA in the treatment of pigment anomaly is not based on the vitamin action, but on the pharmacological one. It is, therefore, used in large amounts for a long period. Little reports are available on the side-effects of the large doses of the vitamin. The experiments of the authors showed, after administering 3 g AsA per day orally or intravenously, no marked increase in the production of oxalic acid in spite of the definite rise in urinary DKA. However, following the dose of 3 g AsA per day for consecutive two weeks and the dose of 9 g per day for three consecutive days the increase in the urinary oxalic acid was marked as compared with the control. It suggests that the decomposition of AsA in the body of man does not necessarily follow the oxidative pathway from DKG to oxalic acid and threonic acid. It is to be noted that over 80% of the vitamin given in large amounts was eliminated as AsA.

When AsA-C^{14} was given intraperitoneally to animals and orally to man, the urinary oxalic acid derived from the vitamin was found to be 20 to 60 and about 10% of the total oxalic acid eliminated in the 24-hour urine of the animals and man respectively, corresponding to 1.5 and 0.16%, respectively, of the AsA administered. Namely, 0.3 to 0.7 mg of oxalic acid was produced from 50 mg of AsA in animals, and 3 mg of oxalic acid from 1.8 g of AsA in man.

However, a daily injection of 0.5 mg AsA for consecutive 17 days resulted in the definite increase of the oxalic acid derived from the vitamin. Attention must therefore be paid to the possible excretion of calcium as oxalate in the urine and the formation of urinary calculi. Burns *et al.* have reported that in their experiments on AsA-deficient guinea pigs oxalic acid accounted for more than 20% of the C^{14} eliminated in the urine. They have also reported that after oral administration of AsA-C^{14} the expired air contained the greatest radioactivity, followed by urine. It isa finding quite different from our observation that the main route of excretion of radioactivity was through the urinary tract, whereas as low as 5% was detected in the expired air. This discrepancy may possibly be due to the difference in the mode of administration. Incorporation of labeled C in organs per g after administering AsA-C^{14} showed high values in adrenals, lungs and bones, possibly indicating the involment of the vitamin in the metabolism of these organs.

SUMMARY

Large amounts of ascorbic acid are recently used in curing pigment anomalies. Investigation was therefore carried out in animals and man on the fate of the vitamin administered in large amounts, especially on the excretion of oxalic acid and the following findings were obtained.

1. After administration of the large amounts of ascorbic acid, it was eliminated in urine mostly in the form of dehydroascorbic acid in the majority of cases (about 80%).

2. When ascorbic acid was administered orally or intramuscularly in a dose of

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3 g per day, there was no marked increase in the urinary oxalic acid. Oral administration of 9 g of the vitamin resulted in an marked increase of the urinary oxalic acid (from 20 to 30 mg).

3. With a rise in the dose of ascorbic acid, urinary diketogulonic acid was increassed. It seems to indicate that the large dose of ascorbic acid promotes the degradation of dehydroascorbic acid into digekogulonic acid, but further oxidation to oxalic acid does not occur markedly.

4. The amounts of oxalic acid-C^{14} detected in the urine of animals and man following the administration of AsA-C^{14} accounted for 1.0 to 1.5% and 0.16% of the C^{14} given in animals and man respectively.

5. Investigation on the effect of an intravenous an injection of a daily dose of 0.5 mg of ascorbic acid carried out for 17 consecutive days on the formation of calculi with a lead ball inserted in the urinary bladder as a nucleus showed that the calculi formed around the nuclei in experimental animals was 7 times as heavy as those formed in the control animals. The amount of oxalic acid contained in the calculi was 0.73 mg as contrasted to 0.33 mg in the calculi of the control animals.

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Studies on the Antidotal Action of Drugs

Part 1 Vitamin C and Its Antidotal Effect against Alcoholic and Nicotine Poisoning

by
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Vitamin C in its various preparations is one of the most commonly administered drugs in dentistry. In connection with many researches related to the poisoning of many pharmaceuticals, research endeavors are also active in the antidotal action of these drugs.

TAMURA, one of the present authors, published his experimental studies on the elimination of an injurious by-effect of promine [1, 2] and also those dealing with leucine against caffeine[3], substances having antidotal effects on morphine and nicotine [4] and anti-alcoholic agent [5].

Although the possibility of vitamin C poisoning has drawn the attention of medical and dental profession for a long time, almost nothing is known about its effect when administered conjointly with some form of amino acid.

In the present report, the authors are concerned with the antidotal mechanism of vitamin C and glucose-cysteine against acetaldehyde responsible for the alcoholism and nicotine, a potent factor in the nicotinism.

Material and Method

By way of experimental animal, male mice of DD strain weighing around 20 grams each were selectively used.

Two kinds of antidotal reagents were prepared: one was a preparation of vitamin C alone where 400 mg (Pharmacopeia japonica) was dissolved in 10 ml pure water and the other contained 50 mg glucose-cysteine and 32.5 mg vitamin C, the latter being referred to as CC reagent here. The CC reagent was thoroughly crushed by the pestle and mortar and 10 ml pure water was added to a proportion of 1 gram.

Similarly, two toxic agents were prepared as follows. One was a 10-time aquous solution of acetaldehyde (Pharmacopeia japonica) and the other was 60 ml physiologic saline in which 17 cigarettes of a common Japanese brand (sold under the tradename of Hilite) were briefly immersed to obtain a nicotine solution.

Experimental Results

1. Antidotal action against acetaldehyde.

The antidotal actions of vitamin C and CC reagent were respectively determined

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by changing concentrations of the latter. Table 1 below summarizes the result under this heading.

Table 1. Antidotal actions of vitamin C and CC reagent against the toxicity of acetaldehyde

Reagent	Acetaldehyde	Manner given	Death rate (%)
Vitamin C alone			
40 mg/kg	10 ml/kg	Given 30 minutes earlier	60
Glucose-cysteine added			
1g/kg		Acetaldehyde and CC together	40
10 mg/kg	10 ml/kg	Given 30 minutes earlier	10
100 mg/kg	10 ml/kg	Given 30 minutes earlier	0
1g/kg	10 ml/kg	Given 30 minutes earlier	0
60 mg/kg	10 ml/kg	Given 30 minutes earlier	20

2. *Antidotal action against nicotine.*

The nicotine solution as described in the foregoing section was injected into the experimental mice and the antidotal action of vitamin C either singly or conjointly with glucose-cysteine was determined (Table 2).

The average length of survival of a mouse after being injected with a nicotine solution was 13.0 minutes.

Table 2. Antidotal actions of vitamin C and CC reagent against the toxicity of nicotine

Reagent	Nicotine (LD 50)	Manner given	Length of survival (Mean)
Vitamin C alone			
400 mg/kg	31ml/kg	Given 30 minutes earlier	16.0 min
Glucose-cysteine added			
600 mg/kg	31ml/kg	Given 30 minutes earlier	10.6 min
1g/kg	31ml/kg	Given 3 days consecutively	20.0 min
1g/kg	31ml/kg	Given 30 minutes earlier	34.0 min

Conclusions

As a result of our research to determine the effect of a conjoint use of glucose-cysteine in connection with vitamin C, the following conclusions were arrived at.

1. When used conjointly with glucose-cysteine, vitamine C will definitely increase its antidotal action against the toxicity of acetaldehyde which is chiefly responsible for the alcoholism.

2. Similarly, a conjoint application of glucose-cysteine and vitamin C has a pronounced increase in its antidotal action against the toxicity of nicotine.

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The History of Vitamin C

Based on an essay which received the Rose Simmonds Memorial Fund Essay Prize for Student Associates of the British Dietetic Association

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SCURVY, THE DISEASE KNOWN to the mariners of old as the calamity of sailors was known for many years before the discovery of the therapeutic value of an ascorbutic factor, vitamin C, found in fresh fruit and vegetables.

Scurvy was not clearly recognised by the Greek and Roman physicians. Hippocrates (*c* 460–377 BC), a Greek physician described an outbreak of sore gums and leg pains amongst soldiers which was probably an outbreak of scurvy.

During the Middle Ages there was a heavy death toll amongst mariners. In 1497 when Vasco de Gama sailed round the Cape of Good Hope scurvy broke out amongst his crew and sixty-two per cent died.

Gerard's Herbal, written at the end of the sixteenth century, describes scurvy – 'Gums are loosed, swollen and exulcerate, mouth stinking, spots on thighs, pale face and body. Swollen feet.' From this description it is easy to visualise the wretched state of the early sailors who suffered from this disease.

The earliest records for the treatment of scurvy are legends from early Dutch writings which tell how Roman legions fell victims of scurvy when they crossed the Rhine. Frieslanders taught them how to cure it with herbs. One of these was probably what later became known as scurvy grass (*cochlearia officinalis*) which under the name lepelbladen (spoon leaves) was used for centuries in Holland to prevent the disease. An account of how the crew of Jacques Cartier's expedition to Newfoundland fell ill with scurvy and were cured by drinking an infusion of leaves from the Ameda tree is found in Hakluyt's 'Principal Navigators' (1600). It was later suggested that the Ameda tree was the local name given to the Spruce fir, the pine needles of which had been well known to the Swedes as a cure for scurvy since the early sixteenth century. About this time the correct treatment for scurvy was probably discovered several times by captains but due to a lack of written records the cure would soon have been forgotten.

The provisions taken on long voyages in the sixteenth century were probably similar to those of Sir Richard Hawkins who set sail to the South Seas in 1593 with beef, pork, brisket and cider. On reaching the Canaries scurvy broke out amongst the crew. He believed that exposure to air prolonged the disease and that an acute form of scurvy was associated with sea air, sea salt, salted meat and fish.

The winter diet of the medieval peasant in England had little protection against scurvy and there is evidence that a prescorbutic condition was common. 'Herbs were given to make loose teeth firm, and for purifying the blood in springtime fresh herbs, strawberry leaves and raw gooseberries were given'. The medical teaching at this time did not recognise scurvy as a nutritional disease. Andrew Boorde in his 'Breviary of Healthe' (1552), treated all manner of sickness and disease but scurvy was not amongst these.

Scurvy became recognised on land as well as at sea, especially amongst communities living together, for example whole monasteries were observed with scurvy. The medical thought of the time was that there are two types of scurvy, one found at sea and the other on land.

In 1734 Johannes Bachstrom, MD, published an eighty-five page book which was well ahead of the current thinking. He demolished the complicated theories that differentiated the types of scurvy and claimed that there was only one disease, resulting from a deficiency of fresh fruit and vegetables.

In 1753 the most comprehensive study of scurvy that has ever been written was produced by James Lind, a Scots naval surgeon. In his treatise, Lind discussed all the records of the disease that had been made and he described the first ever controlled therapeutic trial. This experiment, which was carried out by Lind himself on board the Salisbury at sea in 1747, was an important milestone in the history of dietetics. Lind took twelve patients who all showed signs of scurvy. They all had putrid gums, the spots and lassitude, with weakness of their knees. Their diet consisted of water and gruel sweetened with sugar in the morning. For dinner they had fresh mutton broth and sometimes light puddings of boiled biscuits and sugar. For supper they had barley and raisins, rice and currants or sago and wine. Lind ordered the following additions to their diets. Two of them were given one quart of cider daily; another two were given twenty-five drops of elixir of vitriol (strong sulphuric acid plus aromatics) three times a day on an empty stomach; two tablespoonfuls of vinegar three times a day on an empty stomach were given to another two; two of the worst patients were put on a course of sea water, half a pint daily; two oranges and one lemon daily were given to another two, and the remaining two were given 'the bigness of nutmeg' three times a day. The treatment was carried out over six days.

Lind's observations were that the 'most sudden and visible effects were perceived from the use of oranges and lemons'. One of the patients treated in this way was fit for duty after six days and the other one was the best recovered of the rest. Lind therefore became aware that an adequate supply of fresh vegetables or suitable fruit, i.e. citrus fruit, could cure and prevent scurvy. Lind also emphasised that scurvy had a connection with the life of the sailor. He described the cold damp atmosphere, infection, drunkenness and overcrowding as predisposing causes. Such factors which today we would call 'stress' have only recently been shown to contribute to depleting the suprarenal glands of ascorbic acid.

On Hawkins' salt theory, Lind commented that men working in Polish mines did not suffer from scurvy and that German troops in Hungary had eaten no salt, but had suffered from the disease.

Captain James Cook acted on Lind's advice when he undertook a voyage around the world (1772–75) in which none of the crew developed scurvy. The Royal Navy remained oblivious of the value of Lind's teaching until Sir Gilbert Blane, Lind's pupil, persuaded the Lords of the Admiralty in 1795 to provide a supply of citrus fruits to the Royal Navy.

The nineteenth century was notable in that many voyages were made without a single member of the crew falling ill with scurvy. Ships were provided with lemon juice, preserved by the addition of twenty-five per cent rum. The alcohol was intended to suppress the growth of organisms, but from time to time there were complaints of deteriorated juice that lost its effectiveness. Adulterated lemon [illegible] was probably the cause of the scurvy outbreak on Sir George Nare's polar [illegible]on of 1875.

It was only in 1854 that a Merchant Shipping Act was passed making [illegible] compulsory to provide antiscorbutics to all ships likely to be away from port for more than ten days.

Although scurvy gradually ceased to be an important disease of sailors [illegible] continued to afflict garrisons and civilians of besieged towns and those confined to prisons. The heyday of infantile scurvy was the last twenty years of the nineteenth century when mothers were known to boil orange juice for their infants with the object of sterilising it.

The development of bacteriology in the nineteenth century distracted medical thought away from nutritional diseases and there were subsequently seekers and finders of a 'Bacillus scorbutus'!

This theory led Captain Scott, RN, to ignore Lind's teaching and plan an expedition to the South Pole in 1912 without a single antiscorbutic. This foolish venture had tragic results.

The first important step towards the final isolation of the antiscorbutic factor was made by two Norwegian scientists, Holst and Frölich, in 1907. They discovered that guinea pigs, like man, are susceptible to scurvy when fed diets devoid of fresh fruits and vegetables.

The work that followed was directed at isolating from citrus fruits a substance which, when administered to scorbutic guinea pigs, would cure the symptoms of scurvy.

Citric acid was held by some as the important factor but no therapeutic results were obtained from its administration.

Others postulated that a deficiency of potassium was responsible as this was known to be abundant in fruit and vegetables. When administered as a purified salt potassium failed to cure scorbutic guinea pigs.

There were some workers who were ardent believers of the theory that claimed scurvy to be a bacterial infection. In 1916, Jackson and Moore published the results of experimental scurvy in guinea pigs in which they claimed to isolate after death, a diplococcus from the tissues of a scorbutic guinea pig. They cultivated these organisms in the laboratory and innoculated them into guinea pigs living on a diet that would normally maintain health. They record how they observed the development of haemorrhages in the experimental guinea pigs and from this they adopted the view that scurvy was a bacterial infection.

The first isolation of the antiscorbutic factor came from an apparently unrelated field of biochemistry, that of tissue oxidation. In 1928, while working in Sir Frederick Gowland Hopkins' laboratory, on Redox systems in adrenal glands, Szent-Györgyi isolated a crystalline substance from the adrenal glands, and later from oranges and cabbage though he did not recognise its vitamin properties. In 1932, Glen King, another of Hopkins' students, and his colleague, W. A. Waugh, isolated a substance designated 'hexuronic acid' from lemon juice and they identified this as the substance isolated by Szent-Györgyi four years previously.

'Hexuronic acid', on administration to scorbutic guinea pigs, was found to have properties similar to citrus fruits. 'Hexuronic acid' was renamed ascorbic acid by Szent-Györgyi.

The chemistry of vitamin C

By 1932, Zilva had described the vitamin as a water so[illegible] substance, con-

taining no nitrogen and which was probably a derivative of a hexose sugar. It was known also that ascorbic acid was susceptible to oxidation, especially in alkaline solution.

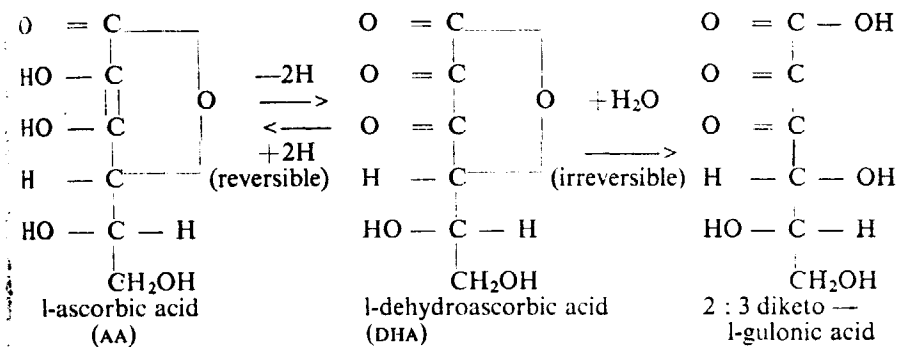
Howarth and Hirst, working in Birmingham laboratories, elucidated the chemical structure of ascorbic acid in 1933.

The synthesis of ascorbic acid was accomplished simultaneously by Reichstein in Switzerland and Howarth in England in 1933. It was synthesised by the action of hydrogen cyanide on l-xylose (obtained from d-galactose).

The biochemical role of vitamin C is linked with its intense reducing action, ascorbic acid being the most active reducing agent known to occur naturally in living tissues.

Ascorbic acid is easily oxidised, especially in an alkaline medium and on exposure to heat, light and trace metals, particularly copper. The first stage of the oxidation to dehydroascorbic acid is reversible, but further oxidation to 2: 3 diketo l-gulonic acid is irreversible.

The Oxidation of Ascorbic Acid



When ascorbic acid was recognised as a reducing agent attempts were made to ascribe to it a function in tissue oxidation.

Szent-Györgyi working in Stockholm won the Nobel Price in 1938 for his study of the biochemical function of vitamin C. His theory stated that vitamin C was involved as a hydrogen carrier alongside flavones, ascorbic oxidase and hydrogen peroxide peroxidase. Many other attempts have been made to determine the precise function of ascorbic acid in tissue oxidation though with little success.

One of the established facts is that the oxidised form of the vitamin (DHA) can act as a hydrogen acceptor in animal tissues in the oxidation of phenolic amino acids, phenylalanine and tyrosine. If l-tyrosine or phenylalanine is administered to scorbutic guinea pigs excessively large amounts of hydroxyphenol derivatives are found in the urine. This can be remedied by giving vitamin C, suggesting that there is a defect in the metabolism of tyrosine and phenylalanine in the absence of vitamin C.

Painter and Zilva (1950) take the critical view that the interaction between ascorbic acid and tyrosine is a physiological response to an unusual situation and has no ir[illegible]tant bearing on normal nutrition. At the present time there is some questio[illegible] whether or not folic acid can replace ascorbic acid in correcting the abn[illegible] excretion of tyrosine derivatives. In any case th[illegible] reaction is prob-

ably not the chief activity of ascorbic acid in the body since only a small fraction of the vitamin is present in the body as (DHA).

Ascorbic acid (AA) is prevented from being oxidised by the presence of glutathione - a dipeptide with a sulphydryl group which can easily be converted from the reduced -SH to the oxidised S-S form. Hopkins observed that, in a system containing glutathione in addition to ascorbic acid and ascorbic oxidase, the glutathione became vigorously oxidised, thus sparing the vitamin. It was also demonstrated by Hopkins and Morgan (1936) that in the reversible oxidation AA ⇄ DHA, glutathione has the property of converting (DHA) back to (AA). This delicate balance between oxidised (DHA) and reduced ascorbic acid (AA) may well be a factor in normal cell metabolism.

The work of Schwartz and Williams (1952) shows how vitamin C is apparently concerned in an enzyme system converting folic to folinic acid (citrovorum factor), and perhaps also in releasing folinic acid from some bound or conjugated form.

A relationship between the adrenal glands and vitamin C was first shown by Sir Robert McCarrison in 1919 when he observed that the adrenal glands in scorbutic guinea pigs are hypertrophied.

The association of ascorbic acid with adrenal cortical hormones became of specific interest in 1948 when the effects of cortisone in rheumatoid arthritis and other diseases affecting connective tissue was discovered. Here were two materials, ascorbic acid and cortical hormones, which influence the mesodermal matrix and are both concentrated in the same gland. However, it was realised that the effect of ascorbic acid on connective tissue is not mediated via the adrenal cortical hormones. Patients whose adrenal glands have been destroyed suffer from Addison's disease but do not develop scurvy. Cortisone was found to have no effects on the scorbutic lesions in guinea pigs (Wolbach and Maddock, 1952). It must also be remembered that the amount of vitamin C in the adrenals is only a fraction of the content of the whole body. There is still no real understanding of the relationship between the adrenals and vitamin C.

Although the precise influence of ascorbic acid on tissue metabolism is not fully understood in chemical terms, the effects of its deficiency on the microscopic structure of tissues was well defined by Wolbach of Harvard, who from 1923 onwards made an intensive study of the tissues in scorbutic guinea pigs. He demonstrated that a deficiency of vitamin C leads to the faulty formation of collagen, part of the intercellular ground substance or matrix that binds together the cells in such mesodermal tissues as capillaries, bone, teeth and connective tissue. Vitamin C was found to be necessary for the maintenance of the normal functional activity of various types of formative cells. Fish and Harris (1934) showed that a deficiency of ascorbic acid leads to the defective production of dentine, enamel and cement. The teeth become loose through insufficient cement substance to bind them to the jaws and a lack of intercellular cement substance in the walls of the capillaries leads to their rupture and to haemorrhages.

The successful healing of a wound requires the formation of strong connective tissue in the scar. The failure of wounds to heal and the reopening of previous scars has long been recognised as a feature of scurvy. Lord Anson observed this amongst members of his crew in his voyage round the world in the *Centurion*, 1740–44.

It was shown by Crandon, Lund and Dill (1940) that the degree of depletion of vitamin C needs to be great in order for a wound to fail to heal. In fact it was only after six months on a grossly deficient diet that a wound fai[illegible] heal normally.

Vitamin C is synthesised in all animals apart from the guinea pig, primates, the Red Indian fruit bat and the red vented bilbul bird. Glucose was shown to be a precursor of the vitamin by Horowitz, Doevschuk and King (1952). They demonstrated that the administration of C^{14} labelled glucose to rats resulted in the excretion of labelled ascorbic acid in the urine. The biosynthetic pathway for vitamin C is d-glucose —> d-glucuronolactone —> l-gulonolactone —> l-ascorbic acid. In rats the synthesis occurs in the liver; in species requiring ascorbic acid in the diet the liver lacks the enzyme necessary for the final step.

No explanation can be given as to why ascorbic acid is found in such large quantities in a diverse set of organs – adrenal cortex and medulla, eye lens, vitreous and aqueous humour and white blood corpuscles – but yet in such meagre amounts in red blood corpuscles, muscle tissue, the brain and pancreas.

Natural sources of vitamin C

Vitamin C has a more limited distribution than the other important water soluble vitamins. Citrus fruits have always been recognised as a valuable source. The value of the potato as an antiscorbutic food was not recognised until the middle of the 19th century when the potato crop failed in 1845 and there was an increased prevalence of scurvy amongst the poor in Ireland.

Chick and Hulme (1917) working at the Lister Institute carried out a comparative study of the value of a number of foodstuffs for protection against scurvy. Using guinea pigs as experimental animals, they soon realised that milk was a poor source. Chick found that 3 cc of fresh orange juice or 2.5 – 5 g of fresh cabbage were equivalent to 100 cc of fresh, whole, unheated milk in antiscorbutic activity.

In the early 20th century the ascorbic acid content of germinating legumes was consciously used to cure scurvy amongst a ship of Greek refugees who were quartered in a League of Nations camp near Constantinople. They induced the germination process by spreading the legumes on wet blankets.

When hard pressed, men have saved themselves from scurvy by curious means. When a case of scurvy arises amongst Eskimos a wild deer is hunted and the sufferer is given the contents of its stomach. There are reports of how Arab camel drivers, towards the end of a long desert journey, may put a stick down a camel's throat to make it vomit. The ejection, which is probably rich in vitamin C because of the vegetable diet, is eaten by the man.

Two of the richest sources of vitamin C are Hungarian paprika and parsley, but because these are eaten in such minute quantities in our diet their value is insignificant.

The superiority of young over old vegetables as sources of the vitamin was recognised in the early 20th century. Hess and Unger (1919) demonstrated the superiority of young over old carrots in preventing scurvy in guinea pigs. They also showed that cooked carrots were less effective than uncooked, and that the water in which they had been cooked was ineffective.

Biochemical studies of ascorbic acid showed that if the oxidation process is continued beyond the stage of dehydroascorbic acid it is irreversible and the vitamin loses its activity. Many plants have been shown to contain an oxidase that facilitates the oxidation process in the presence of oxygen. This en[illegible] to be inactive in the intact plant but is released and becomes activ[illegible] [illegible]les or fruits are damaged by drying, bruising, pounding or cutting [illegible] blunt knife. Oxidation of the vitamin is enhanced, even if the enzyme has been inactivated by heat, in a well oxygenated, alkaline medium and in the presence of copper ions. Ascorbic acid, because of its high solubility in water, is lost from vegetables cooked in excessive amounts of water.

It is evident, therefore, that in order to obtain the maximum vitamin C from its natural sources, fruit and vegetables should be fresh and whenever possible be eaten raw or cooked by 'conservative' methods.

Experimental deficiency in man

Following the work of Lind, citrus fruits became widely used by sailors in the prevention and cure of scurvy though little was known at this time about the human needs for the antiscorbutic factor in citrus fruits.

The first human deficiency experiment on vitamin C was attempted by an Edinburgh trained physician, William Stark, in 1770. Whilst experimenting with various diets on himself he developed unrecognised scorbutic symptoms and eventually died. The tragic result of this experiment meant that other workers were unwilling to repeat it.

It was not until 1936 that this dangerous experiment was undertaken again. Van Eekelen restricted himself to a diet deficient in vitamin C for 84 days and demonstrated that it is possible to subsist for long periods without vitamin C. At the end of the experiment he required 3.2 g of ascorbic acid to saturate his tissues and from this he estimated the daily allowance of vitamin C for a 70 kg man at 60 mg per day.

In 1940, a young American surgeon, John Crandon, fed himself for six months on a diet practically devoid of vitamin C. During the first four months of the experiment he had no signs of deficiency except for a slight loss in weight and a little fatigue. At the end of the six months he demonstrated the unsatisfactory healing of an experimental wound and observed a diminished capacity for work. There were no gross changes of his gums, no anaemia and no significant changes in capillary resistance. On administration of vitamin C all signs and symptoms of deficiency rapidly disappeared. From the amount of vitamin C required to saturate the tissues at the end of the experiment (4 – 6 g) and from the time taken to produce the first signs of scurvy he estimated the daily requirement at between 30 and 45 mg daily.

The most important vitamin C deficiency experiment to be carried out in England was done in Sheffield on twenty subjects. The work was carried out between 1944 and 1946 and was finally compiled as a Medical Research Council Report in 1953 by Bartley, Krebs and O'Brien.

The subjects ate an experimental diet which included 10 mg or 70 mg of vitamin C daily. A control group of ten were given no supplements and in these subjects there was no evidence of scurvy after seventeen weeks.

It is interesting to note that although the plasma concentration of vitamin C fell to that of the deprived group in the group receiving 10 mg vitamin C per day there were no signs and symptoms of scurvy. The group receiving 70 mg vitamin C daily remained in good health throughout the experiment. In the deprived group there was no change in body weight and no increased incidence of infection though colds tended to last longer. Pains, in the back, jo[illegible] and limbs were reported with increased frequency in the depleted volunteers [illegible]rvy developed [illegible] dem[illegible]ated [illegible]'s ex[illegible]ent t[illegible] daily [illegible] vita[illegible]

was not only sufficient to prevent the symptoms of scurvy but was able to remove the clinical features of scurvy. After receiving this dose for 1 – 2 weeks the scorbutic spots faded and within 7 – 9 weeks the skin appeared normal again.

Considered in the light of this experiment, 10 mg vitamin C was the minimum protective dose for the groups under test. To allow for individual variation the recommended daily allowance was set at 30 mg vitamin C.

Recommended daily allowance

When the Merchant Shipping Act was passed in 1854 to provide antiscorbutics to ships there was no scientific knowledge on which to base the allowance.

In 1894, the ration was fixed at 1 oz of lemon or lime juice per head after ten days at sea. The lemon juice would have provided 10 mg vitamin C per day but the lime juice only 4 – 5 mg ascorbic acid.

In 1927, the Board of Trade authorised Masters of Ships to issue concentrated orange juice as an antiscorbutic. The allowance was 0.5 fl oz per head, providing between 30 and 50 mg of vitamin C.

The daily allowances suggested from the results of vitamin C deficiency experiments vary from 30 to 60 mg vitamin C daily, but it was shown in the Sheffield experiment that some individuals can keep fit on as little as 10 mg per day for many months.

There are two schools of thought on the question of how much ascorbic acid an individual needs. Firstly, there are those who believe that a man ought to be fully saturated with the vitamin. Secondly, there are those who maintain that so long as a man is in good health and any small wound heals normally, he is probably getting all the ascorbic acid he requires. Supporters of the first view base their evidence on work done with guinea pigs which undoubtedly require a large intake of ascorbic acid to maintain good health. It has been shown that a guinea pig needs between 5 and 10 mg ascorbic acid to keep in good health, which is enough to cure a patient with scurvy, despite his much greater size.

The League of Nations Technical Commission on Nutrition proposed in 1938 that 30 mg per day was a suitable allowance for an adult. This recommendation received the support of the Sheffield experiment in 1953. The British Medical Association in 1950 recommended 20 mg for an adult, 30 mg for adolescents and 50 mg during lactation. The USA Food and Nutrition Board, believing a state of saturation desirable because of recent evidence that any unexpected injury may create unusual demands for the vitamin, recommended in 1968 a daily intake of 60 mg for an adult.

Current problems

When the knowledge of the importance of vitamin C was established scurvy became a rare disease in this and other developed countries. However, cases of scurvy still occur, particularly amongst two sections of the population – infants and the elderly. Although the prevention of scurvy in infants has largely been accomplished by better education of the mothers and aided by the distribution of chea[illegible] [illegible]ncentrated orange juice, seventy-eight cases of infantile scurvy were reporte[illegible] 60–62 (British Paediatric Association, 1964).

[illegible] fa[illegible] [illegible]elfar[illegible]te ha[illegible] [illegible]ed to find any simple administrative means of preventing scurvy amongst the elderly who are largely unre[illegible]sive [illegible]uca-tion. The distribution of vitamin C tablets has been suggested but this is seldom warranted since vitamin C is rarely the sole nutrient lacking in the diet. The rea[illegible] problem is one of subclinical malnutrition which may be attributed to physical illness, lack of interest in food and a need for a better knowledge and advice on budgeting and of the choice of well balanced meals.

The reality of vitamin C emerged as a result of the early mariners re[illegible]sing that fresh fruit and vegetables were essential for the prevention of scurvy. [illegible] there is the same need for vitamin C in our diet. Although the precise bioch[illegible] role of vitamin C in tissue metabolism is uncertain we know with certaint[illegible] ascorbic acid acts as a reducing agent in the body and is essential for the for[illegible] of collagen. Regarding the desirable daily intake of vitamin C there is still [illegible] controversy, but one fact that cannot be denied is that vitamin C is vi[illegible] health.

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The Vitamin C Requirements in Typhoid Fever

Istvan Telegdi (From the Budapest Szent Laszle Hospital - Senior Medical Superintendant: Prof. Dr. Kalman von Kalocsay)

In the course of extensive studies in the field of avitaminoses and vitamins in general, a whole series of hypovitaminoses was discovered. These are not accompanied by specific symptoms characteristic of vitamin deficiency, but only weaken (reduce) the resistance of the organism and increase the tendency to contract certain types of diseases. It was found that numerous diseases are accompanied to a greater or lesser extent by a partial deficiency of one or several vitamins.

We can distinguish absolute and relative hypovitaminoses. The former include an unsufficient supply of vitamin by the organism, either because the food contains too little vitamin (vitamin-low diet, season, insufficient nutrition) or because the organism cannot make appropriate use of the vitamin present in sufficient amounts. As a result of stomach, intestinal or liver disorders, vitamin absorption is incomplete; vitamin C is split in an anacid stomach, and is again rapidly oxidized in an alkaline gastric juice. Bacteria are also capable of destroying vitamin C prior to its absorption.

A relative hypovitaminosis arises when the vitamin C requirement is increased as a result of certain diseases, so that the otherwise sufficiently high amount of vitamin is no longer capable of satisfying the higher requirements. Feverish diseases increase considerably the vitamin C requirement. A long time before vitamins were known, Noorden already pointed out that it is advisable to supply large amounts of fruits to patients with a high fever. Saturation tests led to the immediate conclusion that the vitamin requirement was increased: in the feverish stage, a saturation of the organism with vitamin C can hardly be achieved. According to Armentano, vitamin deficiency is proportional to the severity of the infection and to the duration of fever.

It is clear that all the above-mentioned conditions responsible for hypovitaminoses are present in typhoid fever. A high fever, a vitamin-low diet, a long duration of the disease, and changes in the intestinal tract are all conditions found in typhoid fever, so that both an absolute and relative

hypovitaminosis must be present and a significant increase of the vitamin requirements must be assumed.

Starting from this trend of thought, many attempts have already been made to exert a favorable effect on typhoid fever with ascorbic acid. In general, a good measure of success has been noted by some authors: the duration of the disease has been shortened, the fever drops, bleeding is less frequent. Other authors, it is true, did not observe any direct effect of ascorbic acid on the fever and the duration of the illness, but also noted that the toxic symptoms are more moderate and the resistance is increased. Good results have been reported by Farkas in our division.

On the basis of these experiences, a systematic administration of ascorbic acid in severe cases of typhoid fever was carried out in our division. The only open question remaining was in what manner and in what amount should ascorbic acid be given, i.e. what dose is sufficient without being wasteful, and whether this dose should be given orally or intravenously.

First, the so-called depot deficiency was established, namely the amount of ascorbic acid necessary to compensate the lack present and to saturate the organism; on the other hand, the daily ascorbic acid requirement was determined, which is necessary to maintain the saturation at a constant level.

As can be seen from the pertinent literature, only Markoff has sofar carried out such quantitative tests in connection with typhoid fever. In light cases, he did not find any depot deficiency, but in severe cases he found a considerable deficiency of 300 to 2000 mg. No information could be found in the literature on the daily dosage necessary to maintain this saturation level. According to Markoff, a daily parenteral dose of 300 mg is not sufficient to keep the ascorbic acid titre at a constant level.

At first, a daily dose of 1000 mg ascorbic acid was given orally. In none of the 6 cases observed by us, could a saturation be achieved in this manner. Even after 12 to 14 days, no vitamin was detected in the urine; the reason for this fact may lie, on the one hand, in the poor absorption conditions, and, on the other, in the vitamin-splitting action of intestinal bacteria. We therefore switched to parenteral administration, and gave ascorbic acid in dry ampoules and as Certascorbin (ascorbic acid solution in ampoules) made by the Certa firm.

The insufficient effectiveness of orally administered vitamin C is also confirmed by the fact that, in contrast to intravenous injection, oral administration does not cause any reticulocytic crisis (Markoff).

Some authors (such as Baumann) are of the opinion that, in saturation tests, the daily dose of 200 to 300 mg ascorbic acid should not be introduced by the intravenous route, since part of the vitamin is excreted by the kidney before it reaches the tissues, whereby a portion of the vitamin is lost without covering up the deficiency. We had no success whatsoever with these doses, and found later that such doses in some cases do not even satisfy the daily requirements. Therefore, we increased the doses gradually up to 1000 mg. According to our experience, the organism can retain considerable amounts of vitamin, when a deficiency is really present. We concluded that saturation takes place when the excretion suddenly showed a sharp increase and when the patient steadily excreted vitamin C over a period of 12 hours. In most cases, both of these phenomena followed a parallel course.

In animal tests, it was found that a large amount of vitamin C lowers the blood pressure. Markoff noted in a patient, after intravenous administration of 600 mg, headaches, chills and collapse; on the following day, the same dose was already well tolerated. In one case, we had to refrain from further administration, since chills, a fever of up to 41°C and collapse occurred after the 15th injection following each injection (patient no. 13).

In case of colicystitis, cystopyelitis and pyuria, no saturation can apparently be achieved, because the colon bacilli (Escherichia coli) split vitamin C (Armentano). In our case of cystitis (No. 14), the daily vitamin C requirement increased from 100 mg to 500 mg, although bacteriological urine findings were negative.

In each case, a systematic thrombocyte count was performed. In 2 cases involving enterorrhagic patients (Nos. 9 and 14), the number of thrombocytes decreased, while in the other cases the initial value increased from 180,000-400,000 to 450,000-600,000.

Our method was the following one: for the determination of ascorbic acid in the living organism, Rotter recommends the intracutaneous injection of n/400 dichlorophenol-indophenol, whereby 2-mm papules are formed. The decoloration times of the dye which has penetrated into the skin provide information on the degree of saturation. For our purposes, this process was found to be not sensitive enough. As already mentioned, our determination of saturation was

based on excretion. Identification in the urine is based on the reducing power of vitamin C. Among the reducing substances present in urine, vitamin C shows the strongest reaction and retains its reactivity also in an acid medium. At pH 2-3, the total amount of reducing substance can be assumed to consist of vitamin C.

For the quantitative determination, the Tillmann method is mostly used, which is based on the reduction of dichlorophenol-indophenol to a leuko compound. We used a modification of this process according to Fischer-Berend. First, the urine excreted 4 hours after injection was titrated; this urine contains practically the entire amount of vitamin which is not bound by the organism (Baumann). The remaining urine portions excreted within 24 hours were acidified with sulfuric acid, collected and titrated.

In the dosage of vitamin C, we followed Widenbauer's method. First, we determined in a blank test the amount of reducing substances without vitamin C; then, we administered large amounts of vitamin daily, until the excretion curve showed a steep rise and excretion lasted for over 12 hours. The amount of vitamin C required for this purpose (or the difference between this amount and the subsequently determined daily requirement) was considered (assumed) as the saturation value. Then, we administered only so much vitamin that the excreted amount of vitamin was only slightly higher than the blank test. The amount needed for this purpose corresponds to the daily requirement.

Widenbauer believes that the numerical result of the blank test is not caused by ascorbic acid even in acidified urine. This opinion is supported by the fact that, even in the most extreme cases of vitamin deficiency when no saturation whatsoever can be achieved, a blank test value can still be detected.

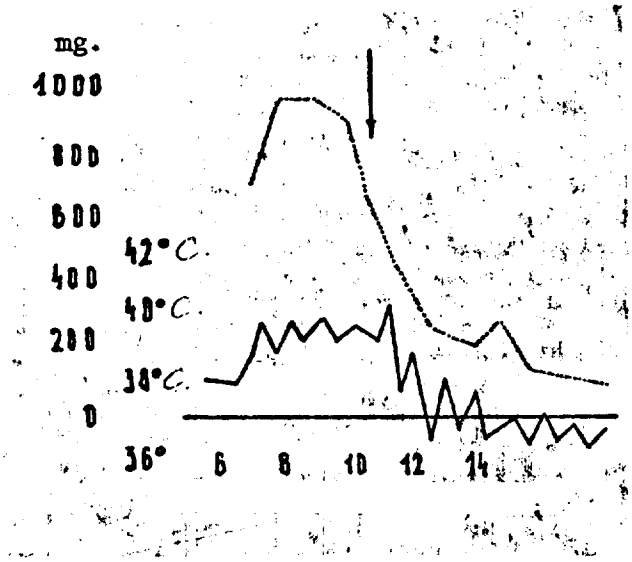
We have observed a total of 14 cases. The severe cases (10 cases) were of primary interest, but, in order to obtain a more general overall view, 4 light cases are also reported here.

Light cases.

1. H. K., 17 years old. Starting on the 7th day of fever, received a daily intravenous injection of 1000 mg ascorbic acid. Saturation after 4 days. (The symbol [↓] refers to the downward pointing arrow in Figs. 1, 6, 7, 8, 9, 12-14.) Deficiency of about 2000 mg. Subsequently, the major part of the administered vitamin was excreted and only 300-100 mg was retained. Since this was a very light case, this large deficit (shortage) must probably be attributed to the deficient and inadequate nutrition and to the simultaneous heavy field work

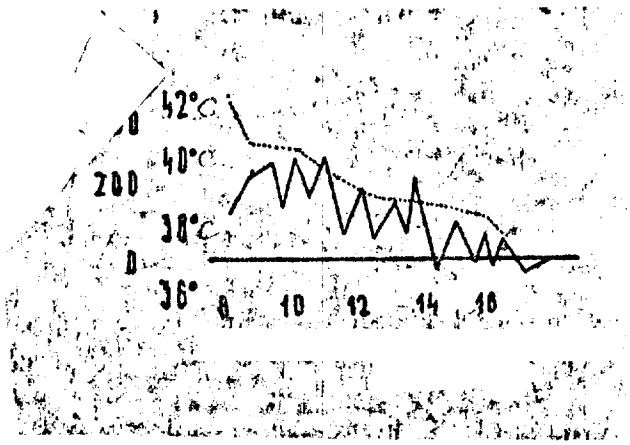
prior to the illness.

Figure 1 -



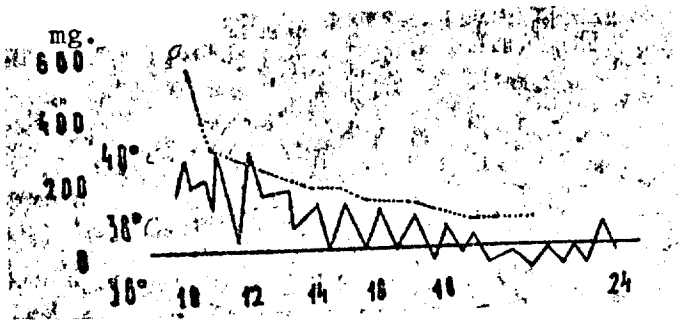
2. H. F., 29 years old, depot deficit (shortage) ϕ (?) daily requirement 300-100 mg

Figure 2 -



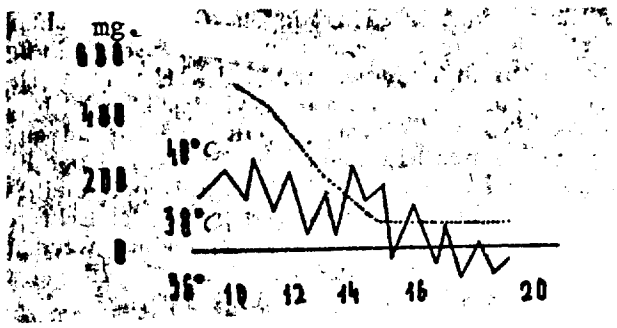
3. S. K., 24 years old, deficiency ϕ (?) consumption 200-50 mg

Figure 3 -



4. F. E., 30 years old, deficiency ϕ (?) requirement 100-30 mg.

Figure 4 -

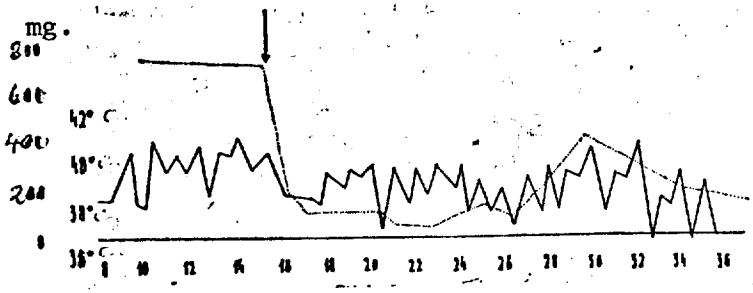


Severe cases. 5. T. I. 24 years old, deficiency ϕ (?), during the high fever period (40°C) requirement about 500 mg, parallel with the drop in temperature gradually less, about 100 mg during convalescence.

Figure 5 -

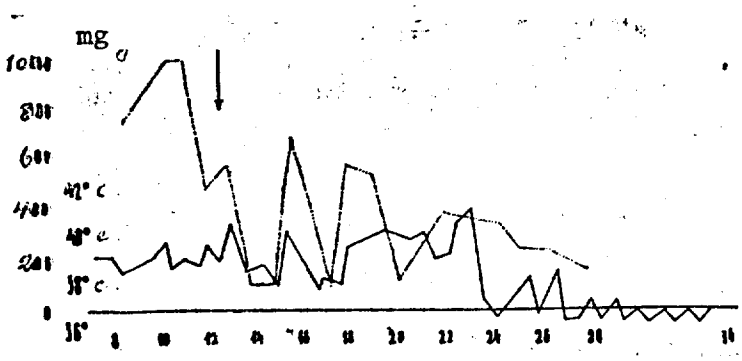
6. H. M., 16 years old. Deficiency 4000 mg. After the latter had been covered, 50-100 mg requirement, after 10 days increased to 100-300 mg with a temperature rise; again 100 mg during the fever-free period.

Figure 6 -



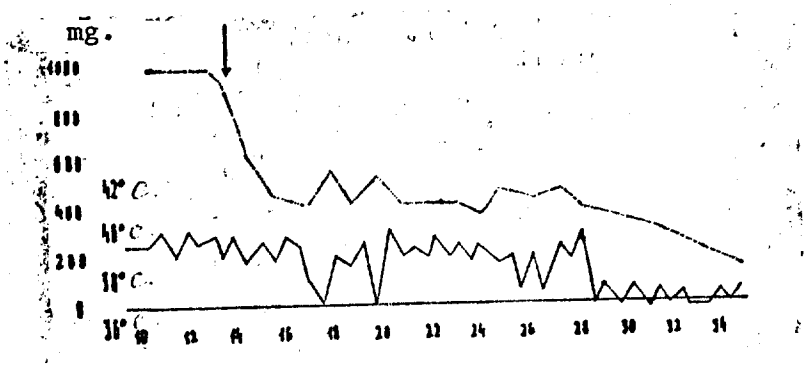
7. K. I., 25 years old, deficit 2000 mg. The variable daily requirement ranges from 100 to 600 mg and runs approximately parallel to the temperature. The urine could not always be collected, and this fact explains in part the fluctuations in the daily requirement; about 300 mg vitamin are retained daily.

Figure 7 -



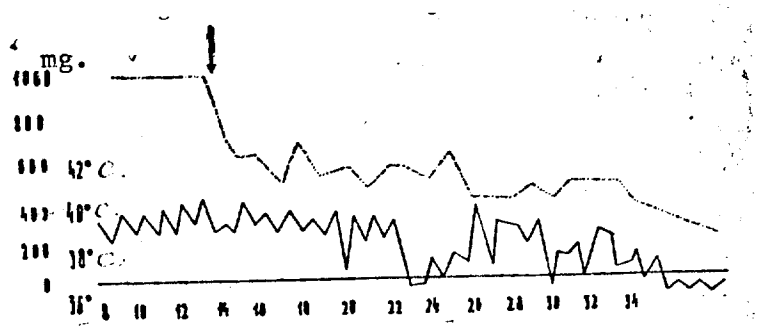
8. N. B., 19 years old, 1800 mg saturation deficit. Daily requirement 400 mg.

Figure 8 -



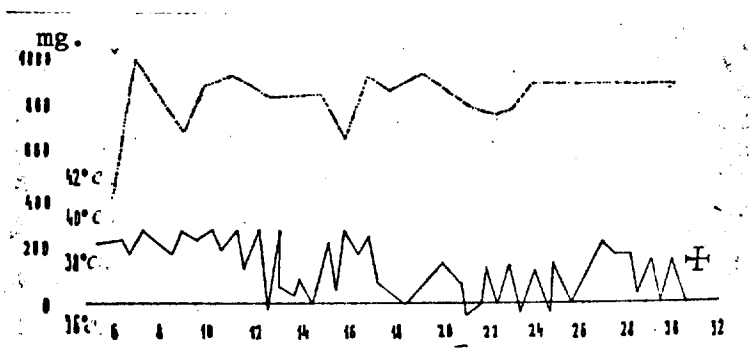
9. B. Z., 28 years old. Deficiency 2500 mg; 500 mg are retained daily. On the 8th day of the illness (hospitalization) 208,000 thrombocytes; this number drops to 90,000 by the 30th day. On that day, 400 cc intestinal bleeding.

Figure 9 -



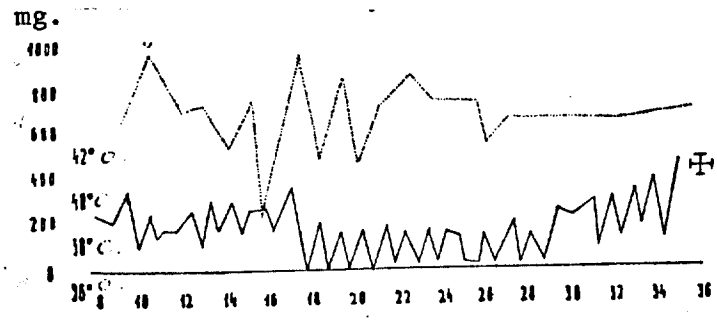
10. H. I., 27 years old. On the 16th day gluteal infiltration, on the 20th day gluteal abscess, on the 26 day exploratory laportomy, died on the 31st day. Diagnosis: gluteal abscess, sepsis, typhoid fever (typh. abd. san.). The patient received 25,000 mg ascorbic acid within 25 days, and only a very small amount of this dose was excreted. Thus, even this large amount was not sufficient to meet the deficit and the daily requirement. On the 6th day of illness, 180,000 thrombocyte; this number gradually increased and amounted to 550,000 on the 26th day.

Figure 10 -



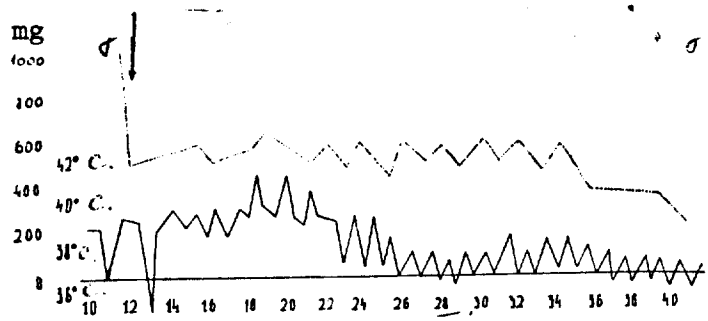
11. A. E., 17 years old. On the 16th day "infiltratio antibrachii" (infiltration of the forearm), on the 23rd day "phlegmone antibrachii" (inflammation of the forearm), incision, died on the 35th day. Diagnosis: sepsis, typhoid fever ("typh. abd. san."). As in the preceeding case, no appreciable excretion could be achieved here. During treatment, the thrombocyte count rose from 210,000 to 400,000.

Figure 11 -



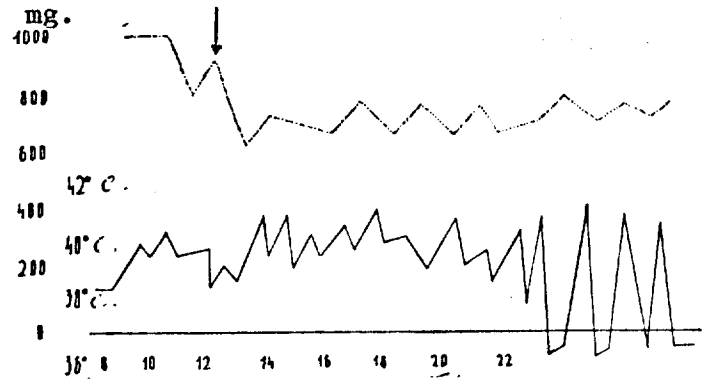
12. B. T., 27 years old. Deficiency 400 mg. Daily requirement, about 600 mg, which remained unchanged even after the fever dropped. Between the 22nd and 35th day, "hydradenitis r. axill. utr." and "carbunculus colli" (neck carbuncle).

Figure 12 -



13. H. Z., 18 years old. Deficit 1600 mg, daily consumption 600 mg. From the 15th to the 22nd day, diffuse bronchitis; on the 18th to 25th day, "otitis media supp. ac. ld." Starting on the 22nd day after injections, chills lasting for 45 to 60 minutes; 41°C fever. The injections were discontinued.

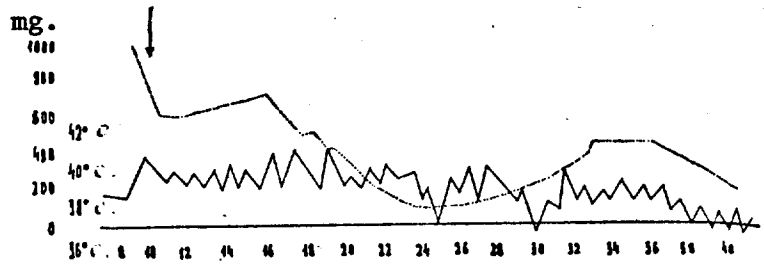
Figure 13 -



14. T. Z., 23 years old. Deficit 300 mg. Daily requirement during the first 10 days, about 600 mg, and only 100 mg during the following week. On the 30th to 38th day, bladder cystitis, with no rise in temperature; the daily amount of vitamin C retained increased to 500 mg during this period. No bacteria were found in the urine. On the 10th day, the thrombocyte count was 250,000, and on the 20th day 150,000. On the following day, 100 cc intestinal bleeding.

During treatment, the toxic symptoms were considerably reduced and the general resistance improved significantly. The temperature and duration of the illness remained unchanged.

Figure 14 -



On the basis of our experience, there is a direct relationship between the vitamin C requirement and the severity of the illness. The depot deficit and daily requirement are much greater in severely ill patients. Every complication increases the daily requirement, and during relapses the daily requirement curve shows a surprising similarity to the fever curve. In light cases there is no depot deficit, and when such a deficit is present, it can only be considered as the result of a hypovitaminosis which is quite independent from the illness (in case #1, 2000 mg). Daily requirement 50 to 150 mg. In severe cases, deficit of (300 up to) 1800 to 4000 mg (in one case, no deficiency). Daily consumption (100 up to) 300 to 500 mg. In case of complications, about 600 mg. In two very severe fatal cases, no saturation could be achieved even upon prolonged dosage.

Summary

The vitamin C deficiency of typhoid fever patients is equal to zero in light cases, and 1800 to 4000 mg in severe cases; the corresponding daily requirement is 50 to 150 mg (light cases), 300 to 500 mg (severe cases), and about 600 mg in case of complications. In two very severe fatal cases, the deficit could not be covered (met) even with 25,000 mg ascorbic acid. During treatment, the toxic symptoms were reduced and the general resistance was considerably improved. The thrombocyte count increased, except in two cases.

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Der Streit der Dogmen ist für uns zu Ende, das große Ringen der Werte hat seinen Anfang genommen.

Alfred Rosenberg.

M...ilung aus dem Szent László-Spital der Hauptstadt Budapest. (Leitender Chefarzt: Dozent Dr. Kálmán von Kaloesay.)

D r Vitamin-C-Bedarf bei Typhus abdominalis

Von Dr. István Telegdi

Im Laufe der eingehenden Untersuchungen auf dem Gebiete der Avitaminosen, beziehungsweise der Vitamine, w...d ...ne ganze Reihe von Hypovitaminosen bekannt. L... ...ehen nicht mit spezifischen, den Vitaminmangel ch... ...sierenden Symptomen einher; sie schwächen nur die Widerstandskraft des Organismus, beziehungsweise er-h...en die Neigung für gewisse Erkrankungen. Es stellte si... heraus, daß zahlreiche Krankheiten mehr oder weniger vom teilweisen Mangel eines oder mehrerer Vitamine begleitet werden.

Wir unterscheiden absolute und relative Hypovitamin...n. Unter ersterer verstehen wir die ungenügende Vitaminzufuhr des Organismus, sei es, weil die Nahrung zu wenig Vitamin enthält (vitaminarme Diät, Jahreszeit, u...enügende Nahrung), oder aber, weil der Organismus d... in genügender Menge vorhandene Vitamin nicht entsprechend ausnützen kann. Infolge Magen-, Darm- oder Leberstörungen ist die Vitaminabsorption ungenügend, im a...ziden Magen wird das Vitamin C gespalten, im alkali-s...en Magensaft wieder schnell oxydiert. Auch Bakterien können das Vitamin C noch vor der Absorption vernichten.

Relative Hypovitaminose entsteht, wenn infolge ge-w...ser Erkrankungen der Vitamin-C-Bedarf erhöht ist, so d... die sonst genügende Vitaminmenge nicht mehr ausreicht. Fieberhafte Krankheiten erhöhen den Vitamin-C-Bedarf erheblich. Noorden wies schon lange vor dem B...anntwerden der Vitamine darauf hin, daß es zweckm...ßig ist, Kranken mit hohem Fieber viel Obst zu verabreichen. Die Sättigungsversuche ließen unmittelbar auf die Erhöhung des Vitaminbedarfes schließen: im fieberhaften S...lium kann kaum die Sättigung des Organismus mit V...min C erreicht werden. Laut Armentano ist der Vit... ...angel der Schwere der Infektion, beziehungsweise der ...rdauer proportional.

Es ist klar, daß alle oben erwähnten Bedingungen der H...povitaminosen beim Typhus abd. vorhanden sind. Hohes Fieber, vitaminarme Diät, lange Krankheitsdauer, Veränderungen im Darmtrakt, so daß sowohl absolute, wie relative Hypovitaminose vorherrschen muß und eine bedeutende Erhöhung des Vitaminbedarfes angenommen werden kann.

Aus diesem Gedankengang hervorgehend versuchten es schon manche, den Typhus abd. mit Ascorbinsäure günstig zu beeinflussen. Die Autoren erwähnen im allgemeinen gute Erfolge: die Krankheitsdauer wird kürzer, das Fieber fällt, die Blutungen werden seltener. Andere sahen zwar keine unmittelbare Wirkung der Ascorbinsäure auf Fieber und Krankheitsdauer, stellten aber ebenfalls fest, daß sie die toxischen Symptome mildert und die Widerstandskraft erhöht. Von unserer Abteilung berichtete Farkas über gute Resultate.

Auf Grund dieser Erfahrungen wurde an unserer Abteilung in schweren Typhus abd. Fällen systematisch Ascorbinsäure verabreicht. Nur blieb die Frage offen, in welcher Weise und wieviel gegeben werden soll, das heißt, welche Dosis genügt, ohne verschwenderisch zu sein, und ob diese peroral oder intravenös zugeführt werden soll.

Vorerst wurde also das sogenannte Depotdefizit festgestellt: jene Ascorbinsäuremenge, die zur Ausgleichung des bestehenden Mangels und zur Sättigung des Organismus notwendig ist; anderseits wurde der Tagesbedarf an Ascorbinsäure bestimmt, die zur Ständighaltung der Sättigung erforderlich ist.

Wie aus dem einschlägigen Schrifttum ersichtlich, hat solche quantitative Versuche bei Typhus abd. bislang nur Markoff angestellt. In leichten Fällen fand er kein Depotdefizit, in schweren aber wies er einen erheblichen Mangel von 300 bis 2000 mg nach. Angaben über die Tagesdosis, mit welcher diese Sättigung beibehalten werden kann, ließen sich im Schrifttum nicht finden. Laut Markoff genügen täglich 300 mg parenteral gegeben zur Konstanthaltung des Ascorbinsäuretiters nicht.

Anfang wurde täglich 1000 mg Ascorbinsäure peroral verabreicht. In keinem der von uns beobachteten Fälle konnte auf diese Weise Sättigung erreicht werden. Auch nach 12 bis 14 Tagen erschien im Harn kein Vitamin C, dessen Grund einerseits in den schlechten Absorptionsverhältnissen, anderseits in der vitaminspaltenden Wirkung der Darmbakterien liegen mag. Also gingen wir auf die parenterale Verabreichung über und gaben die Ascorbinsäure-Trockenampullen, beziehungsweise das Certascorbin (Ascorbinsäurelösung in Ampullen) der Firma Certa.

Für die ungenügende Wirksamkeit des peroral verabreichten Vitamin C spricht auch die Tatsache, daß im Gegensatz zum intravenös verabreichten jene keine Reticulocytenkrise hervorruft (Markoff).

Einige Autoren (wie Baumann) behaupten, daß zu Sättigungsversuchen die Tagesdosis von 200 bis 300 mg Ascorbinsäure intravenös nicht eingeführt werden dürfte, da sonst die Niere einen Teil des Vitamins ausscheidet, bevor dieses noch in die Gewebe gelangt, wodurch ein Teil verloren geht, ohne daß der Mangel gedeckt werden würde. Mit diesen Dosen sahen wir aber überhaupt keinen Erfolg, und später stellte sich heraus, daß sie in gewissen Fällen nicht einmal zur Deckung des Tagesbedarfes genügen. Wir erhöhten deshalb die Dosen allmählich bis 1000 mg. Laut unseren Erfahrungen kann der Organismus — wenn tatsächlich Mangel besteht — recht beträchtliche Mengen des Vitamins zurückhalten. Wir schlossen auf Sättigung, wenn die Ausscheidung plötzlich steil zunahm und Kranke über 12 Stunden hindurch Vitamin C ausschied. Zumeist stellten sich diese beiden Erscheinungen parallel ein.

In Tierversuchen erwies sich eine große Vitamin-C-Menge blutdruckerniedrigend. Markoff sah bei einem Kranken nach intravenös verabreichten 600 mg Kopfschmerzen, Schüttelfrost, Kollaps; den nächsten Tag wurde dieselbe Dosis bereits gut vertragen. In einem Falle mußten wir von der weiteren Verabreichung Abstand nehmen, weil nach der 15. Einspritzung nach jeder Injektion Schüttelfrost, Fieber bis 41° und Kollaps auftraten. (Kranker Nr. 13.)

Bei Colicystitis, Cystopyelitis, Pyurie kann anscheinend keine Sättigung erreicht werden, weil die Bac. coli das Vitamin C spalten (Armentano). In unserem Cystitisfall (Nr. 14) stieg der Vitamin-C-Bedarf von täglich 100 mg auf 500 mg, obzwar der bakteriologische Harnbefund negativ war.

In jedem Falle wurde systematisch Thrombocytenzählung vorgenommen. In zwei Fällen, bei den enterorrhagischen Kranken (Nr. 9 und 14) nahm die Zahl der Thrombocyten ab, in den übrigen stieg der Anfangswert von 180- bis 400.000 bis auf 450- bis 600.000 an.

Unsere Methode war folgende: Rotter empfiehlt zur Bestimmung der Ascorbinsäure im lebenden Organismus die intrakutane Einspritzung von n/100 Dichlorphenol-Indophenol, wodurch Papeln von 2 mm erzeugt werden. Die Entfärbungszeiten des in die Haut gelangten Farbstoffes geben über das Maß der Sättigung Aufschluß. Dieses Verfahren erwies sich für unsere Zwecke nicht genug empfindlich. Unsere Sättigungsbestimmung beruhte, wie bereits erwähnt, auf der Ausscheidung. Der Nachweis im Harn geschieht auf Grund des Reduktionsvermögens des Vitamin C. Unter den reduzierenden Substanzen des Harns reagiert dieses am stärksten und behält seine Reaktionsfähigkeit auch in sauerem Medium bei. Bei pH 2—3 kann die [illegible] Vitamin C angenommen

Stunden nach der Einspritzung entleerte Harn titriert; dieser enthält praktisch bereits die ganze Menge, die vom Organismus nicht gebunden wird (Baumann). Die übrigen binnen 21 Stunden entleerten Harnportionen wurden mit Schwefelsäure angesäuert, gesammelt und titriert.

In der Dosierung hielten wir uns an Widenbauer. Zuerst bestimmten wir im Blindversuch die Menge der reduzierenden Substanzen ohne Vitamin C. Dann gaben wir reichlich das Vitamin täglich, bis die Ausscheidungskurve steil anstieg und die Ausscheidung über 12 Stunden dauerte. Die hierzu benötigte Vitamin-C-Menge (beziehungsweise die Differenz dieser und des nachher bestimmten Tagesbedarfes) wurde als Sättigungswert angenommen. Hiernach gaben wir nur soviel, daß die ausgeschiedene Menge des Vitamins eben nur etwas höher als der Blindwert sein soll. Die hierzu benötigte Menge gibt den Tagesbedarf an.

Widenbauer betrachtet den Blindwert auch in angesäuertem Harn nicht von Ascorbinsäure herrührend. Seine Ansicht wird durch den Umstand unterstützt, daß auch in den äußersten Fällen von Vitaminmangel, wenn Sättigung überhaupt nicht erreicht werden kann, ein Blindwert aufweisbar ist.

Wir beobachteten 14 Fälle. In erster Linie waren die schweren Fälle von Interesse (10 Fälle), aber um eine allgemeinere Übersicht zu gewinnen, wird auch über 4 leichte Fälle berichtet.

Leichte Fälle: 1. H. K., 17 Jahre alt. Erhält vom 7. fieberhaften Tag an täglich 1000 mg Ascorbinsäure i. v. In 4 Tagen Sättigung. (↓) Mangel von etwa 2000 mg. Im folgenden wurde der größte Teil des verabreichten Vitamins ausgeschieden und nur 300—100 mg behalten. Da ein ganz leichter Fall vorliegt, ist dieses große Defizit wahrscheinlich der mangelhaften und unzweckmäßigen Nahrung sowie der gleichzeitigen schweren Feldarbeit vor der Erkrankung zuzuschreiben.

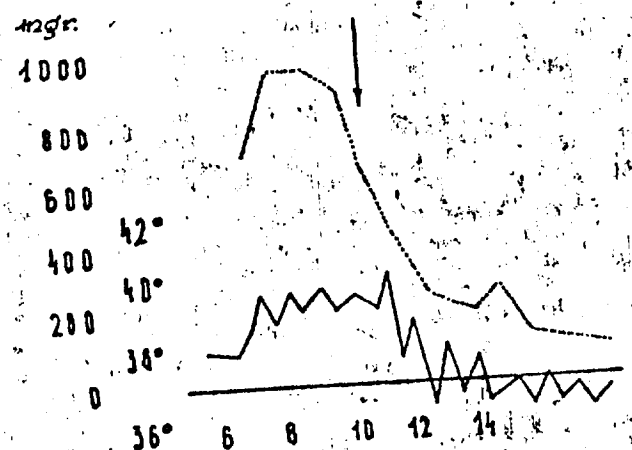
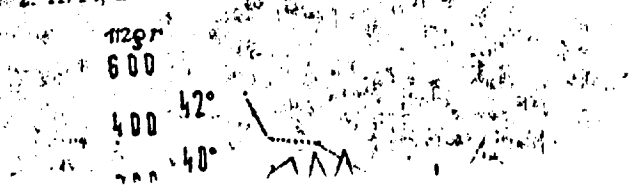
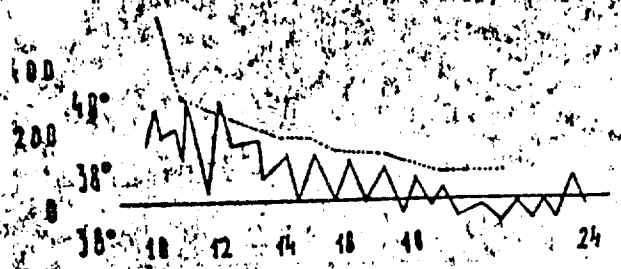


Abb. 1.

2. H. F., 29 Jahre alt, Depotdefizit ∅, Tagesbedarf 300—100 mg



3. K., 24 Jahre alt, Mangel ∅, Verbrauch 200—50 mg.



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4. F. E., 30 Jahre alt, Mangel ∅, Bedarf 100—30 mg.

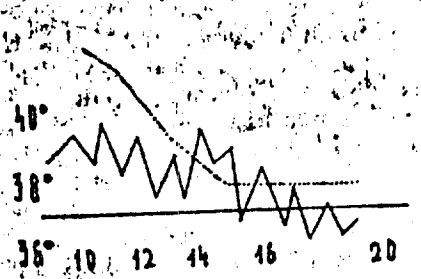
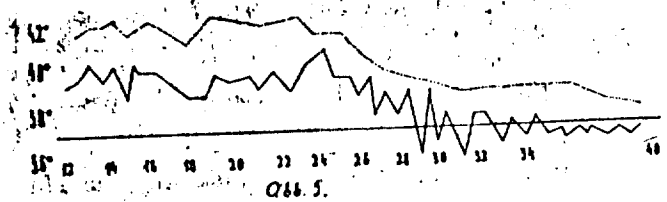


Abb. 4.

chwere Fälle: 5. T. I., 24 Jahre alt. Mangel ∅, während s hohen Fiebers (40°) Bedarf zirka 500 mg, parallel mit dem nken der Temperatur allmählich weniger, in der Rekonvaleszenz



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6. H. M., 16 Jahre alt. Mangel 4000 mg. Nach Deckung dieses —100 mg Bedarf, nach 10 Tagen Erhöhung auf 100—300 mg mit- en eraturanstieg; in der fieberfreien Periode wieder 100 mg.

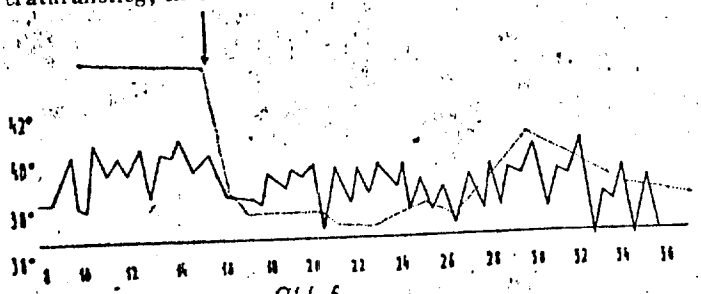
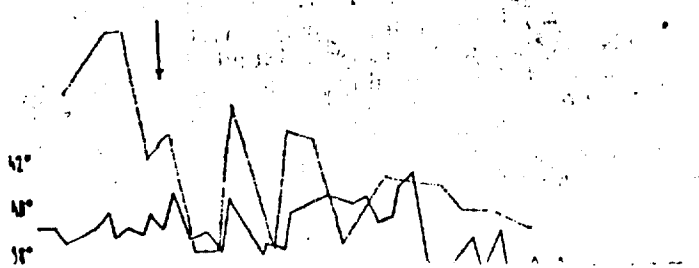


Abb. 6.

. K. I., 25 Jahre alt. Defizit 2000 mg. Der veränderliche Tages- bedarf bewegt sich ungefähr der Temperatur parallel zwischen 0—600 mg. Der Harn konnte nicht immer gesammelt werden, o rch die Abweichungen zum Teil erklärt werden können. Es en zirka 300 mg täglich zurückgehalten.



8. N. B., 19 Jahre alt. 1800 mg Sättigungsdefizit. Tagesverbrauch 400 mg.

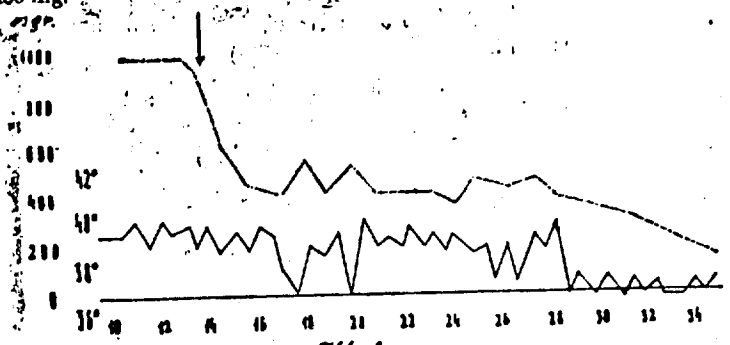


Abb. 8.

9. B. Z., 28 Jahre alt. Mangel 2500 mg. Hält täglich 500 mg zurück. Am 8. Tage der Erkrankung (Aufnahme in das Spital) 208.000 Thrombocyten; die Zahl sinkt bis zum 30. Tage auf 90.000. An diesem Tage 400 ccm Darmblutung.

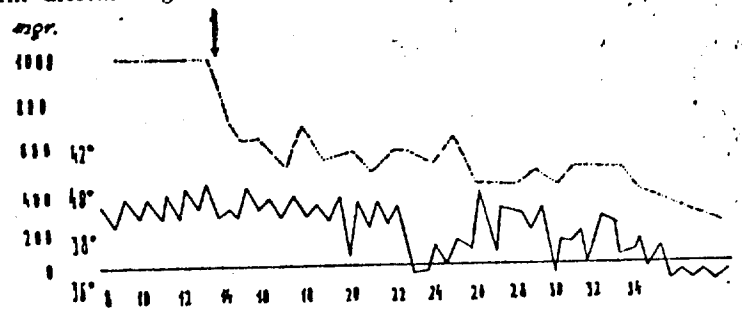
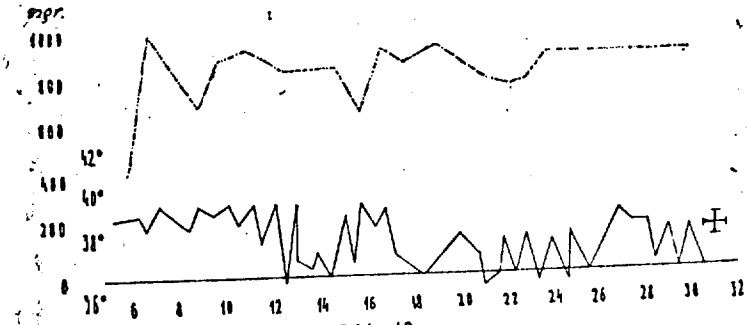


Abb. 9.

10. H. I., 27 Jahre alt. Am 16. Tage Infiltratio glutaealis, am 20. Abscessus glutaealis, am 26. Probelaparotomie, am 31. Exitus. Dg.: Abscessus glutaealis, Sepsis, Ty. abd. san. Der Kranke erhielt binnen 25 Tagen 25.000 mg Ascorbinsäure. Nur ein ganz kleiner Teil dieser wurde ausgeschieden. Zur Deckung des Defizits und des Tagesbedarfes war also auch diese große Menge nicht ausreichend. Am 6. Tage der Erkrankung 180.000 Thrombocyten, allmählich zunehmend, am 26. Tage 550.000.



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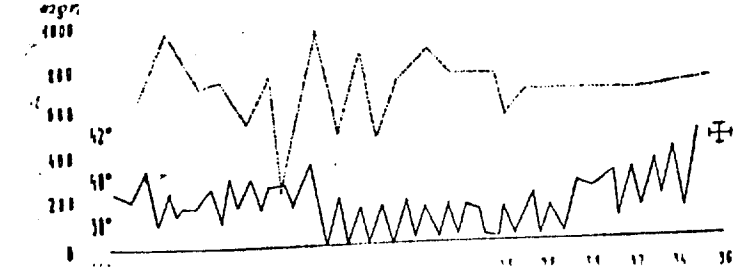
11. A. E., 17 Jahre alt. Am 16. Tage Infiltratio antibrachii, am 23. Tage Phlegmone antibrachii, Inzision, am 35. Exitus. Dg.: Sepsis, Ty. abd. san. Ähnlich wie im vorigen Falle kann auch hier keine nennenswerte Ausscheidung erreicht werden. Während der Behandlung stieg die Thrombocytenzahl von 210.000 auf 400.000.



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12. B. T., 27 Jahre alt. Mangel 400 mg. Tagesbedarf zirka [illegible] mg., der auch nach dem Sinken des Fiebers unverändert bleibt. Zwischen dem 22. und 35. Tage Hydradenitis r. axill. utr. u. Carbunculus colli.

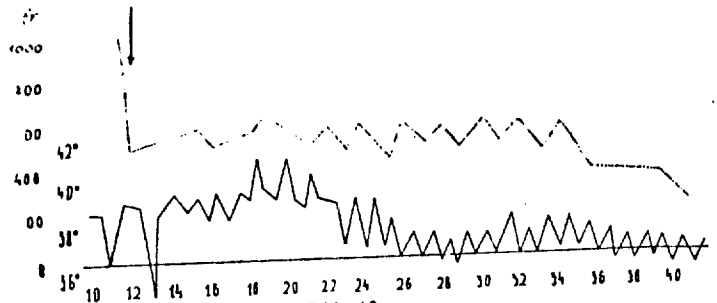

Abb. 12.

Z., 18 Jahre alt, 1600 mg Defizit, Tagesverbrauch 600 mg. Am 15. bis 22. Tage Bronchitis diffusa, am 18. bis 25. Otitis media supp. ac. ld. Vom 22. Tage an nach den Injektionen Schüttelfrost [illegible] bis 60 Minuten lang, 41° Fieber. Die Injektionen werden nicht [illegible]gesetzt.

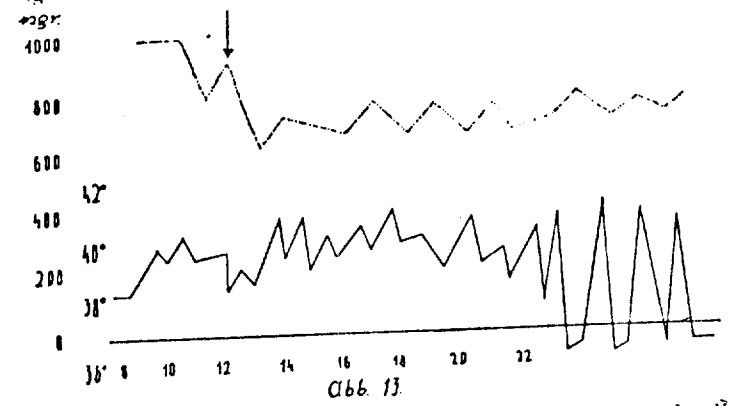

Abb. 13.

14. T. Z., 23 Jahre alt, 300 mg Defizit, Tagesverbrauch die ersten 10 Tage zirka 600 mg, die nächste Woche nur mehr 100 mg, am 30. bis 38. Tage Cystitis vesicae urinariae, und zwar kein Temperaturanstieg, erhöht sich die zurückgehaltene Vitamin-C-Menge auf täglich 500 mg. Harnbefund auf Bakterien negativ. Am 10. Tage 250.000 Thrombocyten, am 20. 150.000. Nächsten Tag [illegible]0 ccm Darmblutung.

Während der Behandlung verminderten sich die toxischen Symptome wesentlich, die allgemeine Widerstandskraft besserte sich bedeutend, Temperatur und Krankheitsdauer bleiben unverändert.

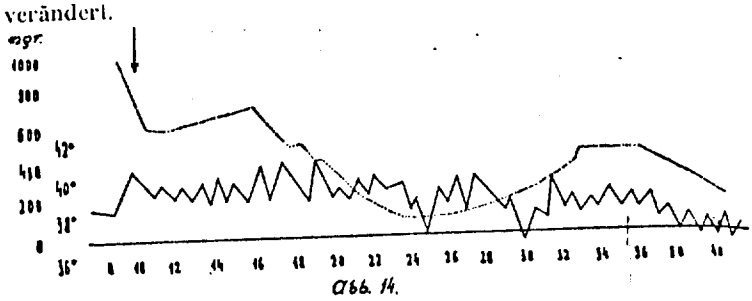

Abb. 14.

Auf Grund unserer Erfahrungen besteht zwischen dem Vitamin-C-Bedarf und der Schwere der Erkrankung unmittelbarer Zusammenhang. Bei Schwerkranken ist das Depotdefizit und der Tagesbedarf viel größer. Jede Komplikation erhöht den Tagesbedarf und bei Rezidiven zeigt die Kurve dieses überraschende Ähnlichkeit mit der Fieberkurve. In leichten Fällen besteht kein Depotdefizit, wenn doch, so kann es nur als Resultat einer von der Erkrankung ganz unabhängigen Hypovitaminose aufgefaßt werden. (In Fall 1 2000 mg.) Tagesbedarf 50 bis 150 mg. In schweren Fällen Defizit von (300 bis) 1800 bis 4000 mg. (In einem Fall kein Mangel.) Tagesverbrauch (100 bis) 300 bis 500 mg. Bei Komplikationen zirka 600 mg. In zwei sehr schweren letalen Fällen konnte auch mit andauernder Dosierung keine Sättigung erreicht werden.

Zusammenfassung. Der Vitamin-C-Mangel Typhöser ist in leichten Fällen 0, in schweren Fällen 1800 bis 4000 mg. Der Tagesbedarf 50 bis 150, beziehungsweise 300 bis 500 mg, bei Komplikationen etwa 600 mg. In zwei sehr schweren letalen Fällen gelang die Deckung des Defizits auch mit 25.000 mg Ascorbinsäure nicht. Während der Behandlung verminderten sich die toxischen Symptome und die allgemeine Widerstandskraft verbesserte sich bedeutend. Mit Ausnahme von zwei Fällen stieg die Thrombocytenzahl.

Schrifttum. Markoff: Deutsche Med. Wochenschr. 1937, S. 131. — Fischer-Berend: Biochem. Zeitschr. 1937, Bd. 291, H. 4—6, S. 221. — Widenbauer: Klin. Wochenschr. 1937, S. 600.

The American Journal of Clinical Nutrition 27: July 1974 pp 719-727

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Fine structural effects of L-ascorbic acid on buccal epithelium[1]

Larry G. Thaete, [2]*M.S. and J. Norman Grim,* [3]*Ph.D.*

ABSTRACT Three groups of guinea pigs were maintained on separate diets. Group I received no ascorbic acid, group II received a dose just adequate for the prevention of scurvy, and group III received a high dose of 100 times the minimum. A portion of the buccal epithelium of these animals was excised after 20 days for the scorbutic group and 55 days for groups II and III. Tissues were then prepared for electron microscopy. Measurements were taken and analyzed statistically for differences in plaque-to-plaque widths of desmosomes. Those in group I were smaller than those in the other two groups. Tonofilaments appeared to be less tensile and/or less frequently attached to desmosomes in group I than in groups II and III. These two observations indicate that ascorbic acid may have a role in the maintenance of the integrity of the tonofilaments and desmosomes in buccal epithelial cells. It is concluded that, for the maintenance of optimum physical integrity of buccal epithelial cells, ascorbic acid may be necessary in larger doses than the minimum required for the prevention of scurvy. It is proposed that doses up to 100 times the minimum may be beneficial. *Am. J. Clin. Nutr.* 27: 719-727, 1974.

Linus Pauling (1) has recently stated that ascorbic acid in large quantities is both beneficial and nontoxic to humans. This has prompted reactions of concern and alarm from some medical scientists (2, 3). Data for further evaluation of these two points of view could reasonably be provided by an electron microscopic approach, but to our knowledge no one has reported on a study of the fine structural effects of varying doses of this compound. This study was directed toward elucidating some of the ultrastructural effects of vitamin C on epithelial cells in the buccal cavity of the guinea pig. Previous studies have shown that the physiological requirements and disposition of vitamin C are similar in humans and guinea pigs, with the exception that the amount of expired CO_2 resulting from ascorbic acid breakdown is lower in man (4, 5). Products of ascorbic acid metabolism excreted in the urine are the same in both species (5). For these reasons, a study of vitamin C in the guinea pig is highly applicable to theories regarding the effects of this vitamin in man. Few studies (6) have been conducted on the effects of large amounts of this vitamin and more information needs to be accumulated that will provide a more solid basis on which to prescribe "safe" and "useful" amounts of ascorbic acid in therapy.

Vitamin C is necessary for the production of collagen (7) and for the formation of the basement lamina (8). Both of these structures are considered important in the attachment of epithelial cells to the underlying lamina propria (connective tissue); thus, they presumably aid in keeping the two tissues bound together as functional units lining the interior and exterior surfaces of the body. If these extracellular products are not synthesized and maintained, the tissues of which they are a part will lose physical integrity. This loss of integrity may, in turn, result in increased susceptibility of these and deeper cells to infection. The correlation that has been shown by some investigators (9) between the ingestion of large amounts of vitamin C and a low incidence of infection could then be the result of increased tissue integrity. This study is designed to compare some of the parameters which may affect tissue integrity in three groups of animals, one without vitamin C in the diet, one on a

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"normal" nonscorbutigenic dose (control group), and a third group receiving a high dose equivalent to that recommended by Pauling (1). It involves an examination of the ultrastructure of the basement lamina, desmosomes, hemidesmosomes, and tonofilaments of germinal epithelial cells in the buccal cavity.

Materials and methods

The experimental animals were 15 guinea pigs (*Cavia* sp.) varying in weight from 200 to 500 g. These animals were divided into three groups of five each; three of the animals in each group were male and two were female. Group I was the scorbutic group (no vitamin C), group II the control, and group III the high-dose group.

An ascorbic acid-free diet for guinea pigs was obtained from Nutritional Biochemicals Corporation in Cleveland, Ohio, and fed ad libitum during the full experimental period of 55 days. Vitamin C solutions were made each day with fresh tap water and pure crystalline powdered ascorbic acid in concentrations of 1 mg/ml and 100 mg/ml. The guinea pigs were weighed weekly to determine the amount of ascorbic acid solution to be administered to each individual. Amounts were measured by pipette and given orally by eyedropper (10).

Group I (the scorbutic group) received only tap water (1.9 to 2.0 ml/animal per day) with no ascorbic acid. Group II (the control group) received 3.8 to 4.0 mg vitamin C/kg body wt per day. This is the minimum amount necessary to prevent the occurrence of the symptoms of scurvy in guinea pigs according to Stare (10) and Rosenberg (11). Group III (the high-dose group) received 380 to 400 mg/kg per day, which corresponds to 2.6 to 2.8 g/day in man and is within the dose range recommended by Pauling (1). Tap water was supplied freely to all animals between feeding periods.

Group I was maintained on the above diet for 20 days at which time definite signs of scurvy were shown by all members of the group. The remaining two groups continued on their vitamin C diets for a total of 55 days before tissues were excised.

To prevent possible alterations in cell ultrastructure by chemical anesthetics, the animals were killed by a blow on the head. Tissue samples were taken from the left cheek in the first molar region of the buccal cavity within 1 min. These were immediately placed into 3% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) and fixed for 1 hr at room temperature. The samples were then cut into thin slices (0.2 to 0.4 mm thick) and fixed for a 2nd hr in fresh glutaraldehyde solution. The tissues were next rinsed in three 5-min changes of buffer and stored at 4 C for 2 to 8 weeks in a buffer solution containing 0.8% sucrose. Postfixation was in 1.0% osmium tetroxide in cacodylate buffer (0.1 M, pH 7.4) for 1 hr at 4 C. After three rinses in the buffer, the samples were dehydrated in 10-min changes of 30, 60, 90, and 100% (two changes) ethyl alcohol and two changes of 99.9% propylene oxide. They were embedded in Bojax epoxy mixture (12) and sectioned with glass knives on an LKB Ultrotome. These sections were mounted on uncoated stainless steel grids and stained in 2.0% aqueous uranyl acetate and Reynolds' (13) lead citrate. Sections were examined with a JEM 7A electron microscope.

One of the parameters examined in this study was the distance between the two attachment plaques of single desmosomes. These were recorded and analyzed statistically for each group. These measurements were made from the outermost (outermost from the center of the cell) border of one attachment plaque, across the cell membrane and intercellular space to the corresponding outer border of the adjoining (second) attachment plaque of the desmosome (see Fig. 3). All measurements were made with a Bausch & Lomb 7× measuring magnifier using a grid with 100 lines/cm. Summary statistics and a t test for significance of numerical results were performed on a Xerox Data Systems 930 computer.

Results

General appearance

At the time of tissue excision, the general macroscopic internal anatomy of the buccal cavity was noted. No obvious differences were observed between groups.

Fine structure

Measurements. For each of the three groups, measurements were made of the distance between the two attachment plaques (see Fig. 3) of 25 different desmosomes. The control group and high-dose group are nearly the same with mean values of 68.5 nm and 65.9 nm, respectively. They are both larger at the 0.05 level of significance than the scorbutic group which has a mean desmosomal width of 58.8 nm (see Table 1).

There are several special structures that occur at the base (proximal end) of the germinal epithelial cells. These were examined, measured, and compared in this study. One of these structures is the basement lamina which contains several structural layers. There is an electron transparent region (lamina lucida) immediately outside the plasma membrane at the base of the germinal cell. The second layer is an electron opaque zone (lamina densa) and is adjacent to the lamina propria (connective tissue). These two zones are of equal thickness. Hemidesmosomes are observed along the basal cell membranes. These have only one attachment plaque located along the membrane at the proximal end of the cell. The position that would ordinarily be occupied by a second attachment plaque on the membrane of a neighboring cell is taken up by the lamina

FIG. 1. Basement lamina region (proximal end of germinal cells) typical of all groups. Lamina densa (ld), lamina lucida (ll), caveolae (c) and hemidesmosomes (h) are all visible. Other prominent structures include collagen fibrils (f), tonofilaments (t) and the basal cell membrane (cm). ×40,000. Inset is a higher magnification view of typical caveolae (c), representative of those seen in each group. ×50,000.

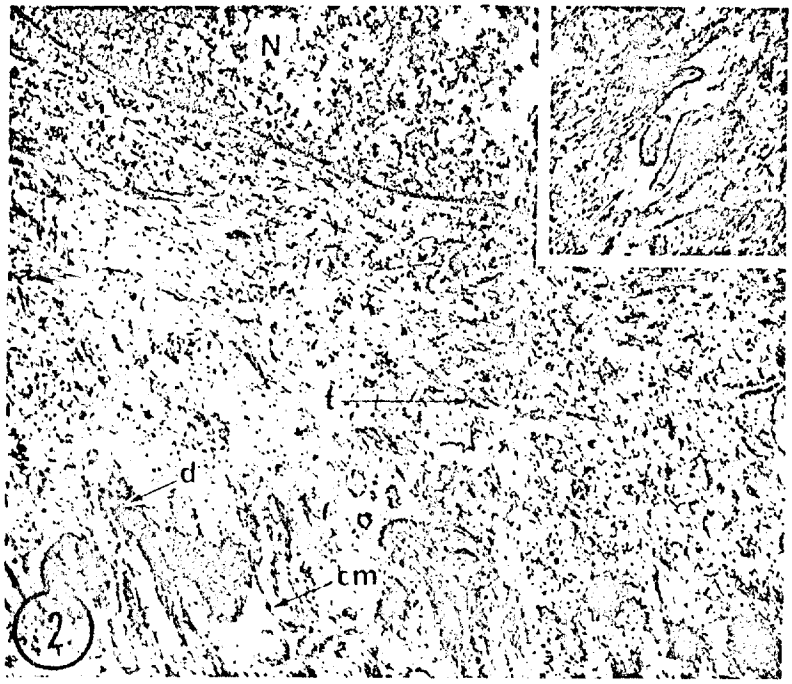

FIG. 2. Typical view of a portion of a germinal epithelial cell of group I. Note the free ribosomes and the randomly scattered bundles of tonofilaments (t). Other labeled organelles include the cell nucleus (N), desmosomes (d), and interdigitated cell membrane (cm). ×20,000. Inset is an example of the enlarged intercellular spaces (arrow) often observed in this group. ×30,000.

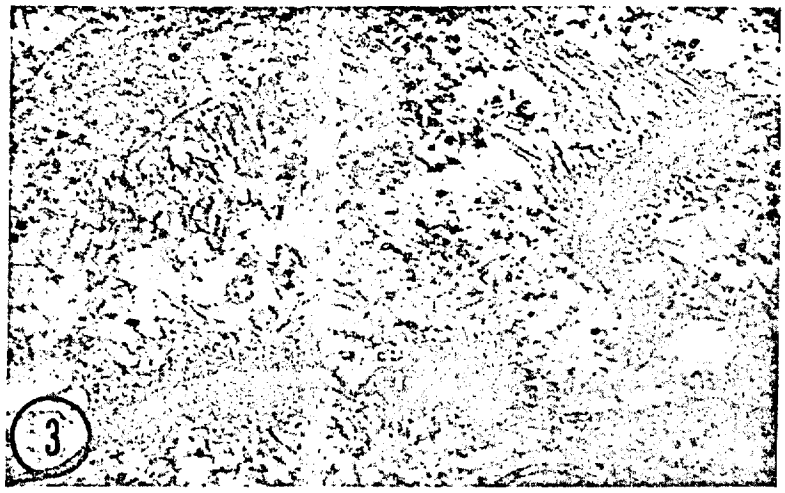

FIG. 3. High magnification of typical desmosomes in group I. Note the fragmentary appearance of the tonofilaments (t) as though they are short segments or are following a tortuous path as they extend from the attachment plaques (p) of the desmosomes. The length of the white bar between attachment plaques of one of the desmosomes demonstrates the plaque-to-plaque distance measured for statistical analysis of the desmosomal widths. ×75,000.

TABLE 1
Summary statistics for desmosome widths

Ascorbic acid dose, mg/kg/day	No. measured (per group)	Mean ± SEM, nm	Mode, nm	Range, nm	SD, nm	Significance[a]
0	25	58.8 ± 1.1	60.0	46.7–66.7	5.3	Group I different
4	25	68.5 ± 1.4	66.7	60.0–80.0	6.8	from II
400	25	65.9 ± 0.8	66.7	60.0–73.3	4.0	and III

[a] $P = 0.05$.

densa. In all groups, an electron opaque structure is present inside the cells and beside and parallel to each hemidesmosome attachment plaque (Fig. 1). Each of the constituents noted above is present with the same apparent structural features and dimensions in all three groups, although there is considerable variation in their size and distinctness within each group. All groups display prominent caveolae ("c" in Fig. 1), which are located only along the basal membranes of basal epithelial cells. These are microscopic vesicles, pinocytotic-like in appearance, and with diameters of approximately 50 to 150 nm.

General appearance of organelles. The germinal cell cytoplasm of the scorbutic animals contains large numbers of free ribosomes and tonofilaments randomly scattered in small bundles (Fig. 2). The tonofilaments that extend from the attachment plaques of desmosomes most often appear as short fragments, though there are occasional regions where parallel "bundles" appear (Fig. 3). Intercellular spaces are often expanded, compared with other groups (Fig. 2, inset).

Buccal cells from organisms of the control group (II) have a marked concentration of microgranules (Fig. 4). These are electron dense bodies distributed throughout the cytoplasm of the epithelial cells. Most of them appear as spherical organelles and range in size from approximately 50 to 250 nm in diameter. They do not appear to be bound by membranes (see Fig. 4), although it is possible that a limiting membrane could be masked by their opacity. The germinal epithelium of this group contains many more of these granules than the other two groups: in fact, microgranules are rarely visible in tissues from groups I and III. A view of typical desmosomes as seen in most

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sections from group II is shown in Fig. 5. Although not sharply defined, the tonofilaments still often appear as short segments, the same as has been noted previously from group I. Some polyribosomes are visible in group II cells but they are not present to any marked degree.

Polyribosomes are often seen in group III cells (Fig. 6, inset). Tonofilaments are more densely distributed throughout the cells of this group than those of the other two. After observing many sections from many tissue blocks we are impressed by the large number of tonofilaments seen extending from attachment plaques of the desmosomes for longer distances in the plane of sectioning than has been noted for groups I and II (compare Figs. 2, 4, 6 and 7). These results are notably uniform throughout the specimens examined.

Both smooth and rough endoplasmic reticulum are present only in short and infrequent segments in each group. For a summary of the preceding data see Table 2.

Discussion

Our ultimate interest in this study was to help to elucidate further the role of ascorbic acid in man. As this research was not conducted at a medical facility, human volunteers were not available. The guinea pig was considered a relevant substitute, as previous investigations have shown that experimental results concerning vitamin C in the guinea pig correspond well with similar experiments in the human (5).

Desmosomes, hemidesmosomes, and tonofilaments were the structures primarily examined in this study because there is evidence that these organelles give the buccal tissue some physical support by holding it together as an integral unit. Evidence to support this concept comes from the work of Charles and Smiddy (14) who studied the hemidesmosomes and tonofilaments in human epidermis. It was discovered that in specimens of stretched skin, the hemidesmosomes appeared to have been pulled inward toward the centers of the basal

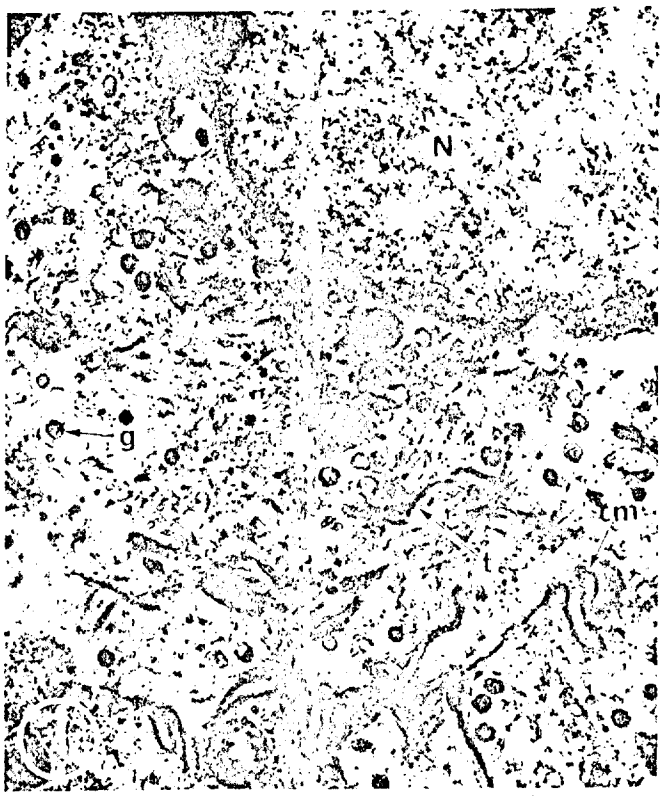

FIG. 4. Typical view of part of a germinal epithelial cell of group II. Note the high concentration of microgranules (g) throughout the cytoplasm. Other organelles include ribosomes, tonofilaments (t), cell membrane (cm) and nucleus (N). X 20,000.

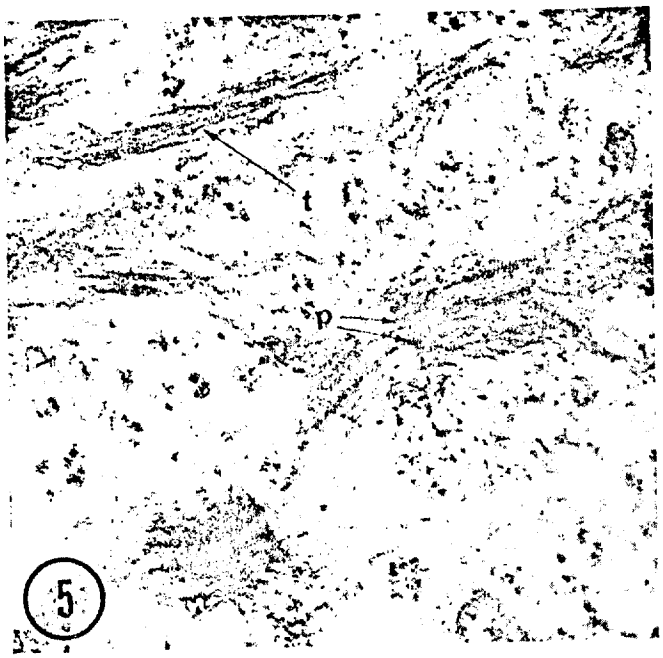

FIG. 5. High magnification of typical desmosomes in group II. The tonofilaments (t) are somewhat indistinct but show little difference in configuration from those in group I (Fig. 3). Only short segments are seen extending from the attachment plaques (p), indicating fragmentation or winding in and out of the plane of sectioning. This view of desmosomes is typical of the material in this group. ×75,000.

FIG. 6. Typical view of the germinal epithelial cells of group III. Note the greater overall density of tonofilaments (t) than that found in groups I and II. Also present are rough endoplasmic reticulum (ER), desmosomes (d), cell membrane (cm), and nucleus (N). ×15,000. Inset demonstrates the tonofilament bundles and both free and attached ribosomes. Some ribosomes are clustered into polyribosomes. ×25,000.

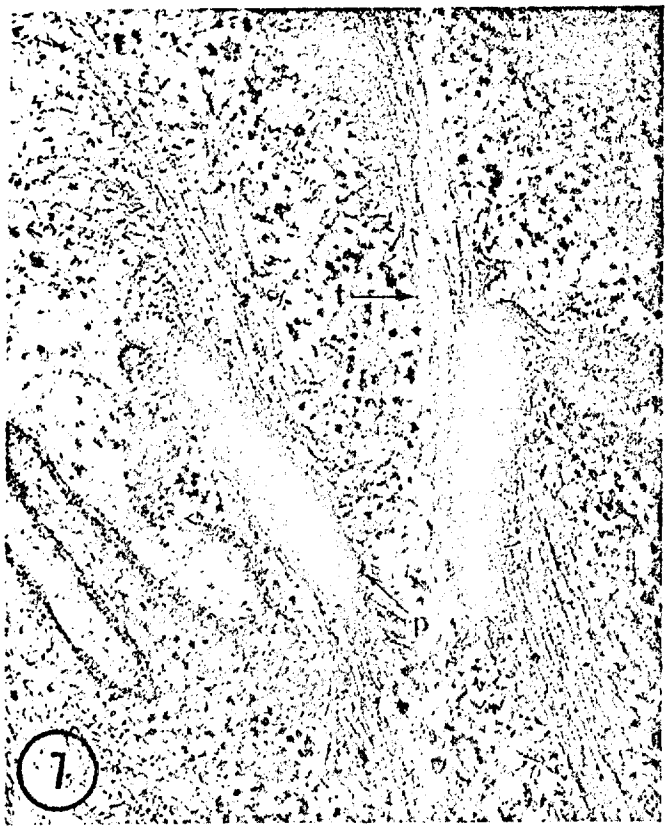

FIG. 7. High magnification of typical desmosomes in group III. The tonofilaments (t) appear to extend as parallel arrays for longer distances away from the attachment plaques (p) in the plane of sectioning than has been noted for groups I and II (Figs. 3 and 5). They may be less fragmentary or follow a more direct pathway from the plaques than those of the other two groups. ×60,000.

TABLE 2
Summary of results

Parameters	Group averages		
	I	II	III
Width of desmosomes, mean	58.8 nm	68.5 nm	65.9 nm
Basal lamina	No differences noted between groups		
Tonofilaments	Random, appear as short segments	Similar appearance as in group I	More densely distributed, fairly long segments
Ribosomes	Free	Some polysomes	Numerous polysomes
Microgranules	Few	Many	Few

cells. Stern (15) discusses their results, extends his analysis to include the desmosomes and argues convincingly that the hemidesmosomes hold the epithelium to the connective tissue (lamina propria) while the tonofilament–desmosome system (with attachment plaques of adjoining epithelial cells presumably "cemented" together) serves to distribute stresses throughout the epithelium and hold it together as a unit. According to this model, when the cells are pulled or stretched, the tonofilaments pull on the attachment plaques of the desmosomes and hemidesmosomes to which they are securely fastened. This would be expected to cause the distance between attachment plaques and the width of the lamina lucida and perhaps the lamina densa to become slightly greater than when no tension is applied.

As noted earlier, the distance between attachment plaques of adjacent cells is smaller in the scorbutic group than in groups II and III. Tonofilaments in this group appear as short segments or as though following a tortuous route through the cytoplasm. If tonofilaments distribute stress in a tissue as suggested by Charles and Smiddy (14) they may, when properly connected, exert a constant tension on the attachment plaques of the desmosomes. If, as is possibly the case for the scorbutic group (I), some or many of these connections are absent or if the tonofilaments are distorted, broken, or follow a winding route, the plaques may be allowed to move closer together. This is in fact the case for group I. The desmosomes for group II are of nearly the same width as those in group III (Table I). These data lead one to specualte that the tonofilament-desmosome complexes may be more tensile or intact in groups II and III than those in group I. The numerous groups of straight tonofilaments seen in group III (Fig. 7) supply added evidence for the possibility that there is increased tension on these structures in animals receiving ascorbic acid. One could then theorize that vitamin C has a role in maintaining proper integrity and orientation of the tonofilaments themselves.

The fact that there is no significant difference in desmosome width between groups II and III (Table I) is not completely resolved at this time and requires further comment. Perhaps there is a sufficient number of intact tonofilaments in group II tissues to hold the attachment plaques away from each other at a similar distance to that found in group III while the "better" oriented filaments in the latter group add extra strength, which may not be demonstrable under the conditions (unstressed) of this experiment.

The electron transparent region of the basement lamina (lamina lucida) contains an unidentified homogeneous fluid or gel which, according to Stern (15), "glues" the basal cell plasma membrane to the adjacent tissue. Priest (8) studied mouse parietal yolk sac and found that the synthesis of epithelial basement lamina is dependent on the presence of vitamin C. Pierce et al. (16) report that the basement lamina is produced by the endoplasmic reticulum of the basal cells. They conducted an in vivo study using ferritin-labeled antibodies for basement lamina and found them localized in the endoplasmic reticulum of the basal epithelial cells. In an excellent review of the literature, Frithiof (17) has also revealed evidence that verifies that the basement lamina is of epithelial origin and can be formed without contact with the underlying lamina propria. Stern (15) has reported the presence of minute vesicular invaginations, or caveolae, of the plasma membrane along the basal surface of the basal cells. He suggests that these may be related to vesicular transport and one may speculate that they function in the movement of materials toward the basement lamina, ultimately for its production. The function of the caveolae cannot, however, be theorized with any degree of certainty on the basis of static micrographs. In any case, the results of the present experiment indicate that vitamin C is not necessary for the formation of the caveolae. Because the basement lamina appears to be similar in all three groups, vitamin C does not seem to be necessary for its maintenance during the experimental period of 20 days.

As ascorbic acid is essential in the formation of several extracellular structures that are thought to be involved in the physical integrity of a tissue (e.g., collagen and lamina densa), it is possible that the vitamin also has a previously undetected role in the synthesis of intracellular constituents and/or the interattachment of these structures to form integral units within the cell. One is inclined to speculate that this vitamin is necessary for the formation of tonofilaments or their attachment to desmosomes and hemidesmosomes. A role such as this would explain the differences for tonofilaments observed between groups. The results of this experiment suggest that vitamin C may be advantageous in larger amounts than those recommended for the prevention of scurvy to enhance the integrity and function of the tonofilament–desmosome systems within the buccal epithelial cells.

Silverman (18) has studied electron opaque microgranules similar to those seen in this study. He has compared them in an ultrastructural study of the cytoplasm of basal and parabasal cells from normally and abnormally keratinized human buccal epithelium. They are thought to play a role in the secretion of intercellular substances during the keratinization process. However, the significance of these organelles cannot be interpreted from the data

accumulated in the present study. This phenomenon needs further study with doses of ascorbic acid intermediate to those used in this experiment.

It is not possible to say with assurance what the direct cause is for the enlarged intercellular spaces in group I. One could speculate that they may be due to decreased production of cementing substances between cells, with a resultant separation of adjacent membranes. This possibility is suggested by the evidence that ascorbic acid is involved in the formation of structures which influence the physical integrity of tissues and may therefore be involved in the synthesis of intercellular cementing materials. The spaces could also be the result of a partial degradation or lack of replacement, or both, of some of the previously existing cementing materials due to a lack of vitamin C. Another possible explanation is the presence of an osmotic imbalance in the tissues. If vitamin C is directly or indirectly responsible for the tonicity either within these cells or in extracellular fluids, its absence might alter tonicities sufficiently to cause the inside of the cells to become hypotonic and thereby shrink as water is lost from them into the intercellular spaces. The latter would then widen with the increased volume of extracellular fluid. This idea is, of course, highly theoretical.

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The determination of vitamin C in evaporated and fortified sterilized milks

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Summary. A study has been made of the use of 2,6-dichlorophenolindophenol (DCP) and also of 2,4-dinitrophenylhydrazine (DNPH) with and without chromatography of the DNPH derivatives, for the determination of ascorbic acid (AA) and dehydroascorbic acid (DHA) in evaporated milks, and of AA in AA-fortified sterilized milks.

In the DNPH method, when interfering compounds were removed by the chromatographic separation of the DNPH derivatives, absorption curves typical of the pure DHA derivative were obtained.

The DCP method gave erroneously high values for the AA and DHA content, and the lower values obtained by the DNPH method with chromatography of the DNPH derivatives were judged correct. Thus for an evaporated milk, the values for vitamin C content (AA + DHA) were, in mg/100 ml reconstituted milk, 0·88 (DCP method), 0·66 (DNPH method without chromatography), and 0·48 (DNPH method combined with chromatography). The AA contents (mg/100 ml) of the 2 fortified sterilized milks were 3·63 and 5·78 (DCP method); 3·01 and 4·28 (DNPH method combined with chromatography).

The AA contents of the 5 different evaporated milks, determined by chromatography of the DNPH derivatives, ranged from 0·07 to 0·63 mg/100 ml reconstituted milk. The DHA content was negligible.

As judged by the shape of the absorption curves after chromatography of the DNPH derivatives, it is concluded that this method is the most reliable of those studied.

The use of 2,6-dichlorophenolindophenol (DCP) and of 2,4-dinitrophenylhydrazine (DNPH) for the determination of vitamin C [ascorbic acid (AA) + dehydroascorbic acid (DHA)] in milks given various heat treatments has been studied by Toothill, Thompson & Edwards-Webb (1970). The intensive heat treatment used in the preparation of evaporated and sterilized milks may lead to the formation of compounds with free sulphydryl groups and of reductones, which may interfere with the determination of vitamin C by simple oxidation–reduction titration procedures, as for example with DCP, causing the results to be erroneously high. Although the specificity of the DCP procedure can be improved to some extent, this reagent is essentially unspecific. DNPH, which reacts with carbonyl compounds, is more specific and forms an osazone with DHA after the transformation of the DHA to 2,3-dioxogulonic acid (DOA). Treatment of the osazone with sulphuric acid (Roe & Kuether, 1943) gives a red coloured solution with an absorbance peak at 522–526 nm. Other carbonyl com-

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pounds, including reductones, may interfere but the specificity of the method can be greatly improved by the chromatographic separation of the DOA osazone from other DNPH derivatives (Gordon & Noble, 1959; Mapson, 1961; Vuilleumier & Nobile, 1962; Strohecker & Henning, 1965; Association of Vitamin Chemists, 1966). AA reacts with DNPH only after oxidation to DHA, and with the inclusion of a reduction step the method can be used to determine AA, DHA and DOA (Roe, Mills, Oesterling & Damron, 1948).

The method of Roe *et al.* (1948), which does not include chromatography, was used in a modified form by Toothill *et al.* (1970) and was found to be unsatisfactory for strongly heated milks, since although the calculated results indicated the presence of moderate amounts of AA, the absorption curves of the DNPH derivatives showed no evidence of this. Because Toothill *et al.* (1970) found the method of Roe *et al.* (1948) to be specific for the determination of vitamin C in in-bottle sterilized milk only after the chromatographic separation of the DNPH derivatives, it was felt that the investigation should be extended to include evaporated and AA-fortified sterilized milks. The results of this study are now reported. For brevity the DNPH derivatives are referred to as hydrazones.

EXPERIMENTAL

Milks

Evaporated milks (unsweetened). Five different proprietary brands (Evap. A–E) purchased from local shops were kept in a cool room and opened within a few days, except for Evap. Bb, which was stored unopened at about 3 °C for 6 weeks. Evap. Ba and Bb had the same batch number.

Sterilized milks. These were a proprietary full-cream (Ster. A) and half-cream (Ster. B) baby-food formulation, supplied by the manufacturer immediately after processing. These milks contained added vitamin C, vitamin D and ferrous iron. The sugar content had been raised to about 7 % with sucrose (Ster. A) or lactose (Ster. B). The heat treatment, to which they had been subjected, included in-bottle sterilization at 116 °C for 10 min followed by incubation at 32 °C for several days. Before analysis the milks, which were in clear glass bottles, were kept for 64 days in the laboratory at ambient temperature and in the light, about 4 m from a shaded, south-facing window. The oxygen content of samples opened on the first day of storage was measured and found to be < 1 ppm (J. E. Ford, personal communication).

Analysis

Details of the methods used are given by Toothill *et al.* (1970). The evaporated milks were reconstituted with distilled water and samples were taken immediately for analysis.

DCP method. The titrations were carried out on metaphosphoric acid extracts of the milks. Bromine was used as the oxidizing agent and hydrogen sulphide as the reducing agent.

DNPH methods. Diluting Solution (Toothill *et al.* 1970) was added to the extracts of the sterilized milks to give solutions with a suitable concentration of AA.

For determinations without chromatography, aliquots of standards and of untreated, oxidized and reduced milk extracts were coupled with DNPH for 3 h at 37 °C. With Evap. C, additional aliquots were coupled for about 19½ h at room temperature.

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The absorbance was determined at 524 and 540 nm with 42 % sulphuric acid solution in the reference cell, 40 min after the addition of 85 % sulphuric acid solution to the hydrazones for colour development. Blanks were also determined. Immediately afterwards, the absorption spectrum was determined, first with 42 % sulphuric acid solution and then with the appropriate blank in the reference cell, using a recording spectrophotometer over the range 400–600 nm. At the same time as aliquots were taken for the 3-h coupling period, additional aliquots were removed for the chromatographic procedure and, for convenience, were coupled at room temperature (about 20 °C) overnight for a total of about 19½ h. Because of the long time required for the analysis, the column eluates were stored overnight at −30 °C and the final separation of the DNPH derivatives by thin layer chromatography was carried out on the next day. The absorption spectrum of the osazone was determined over the range 300–600 nm with 42 % sulphuric acid solution in the reference cell, 40 min after the addition of 42 % sulphuric acid solution for colour development.

Rubber gloves were worn to avoid contact with DNPH.

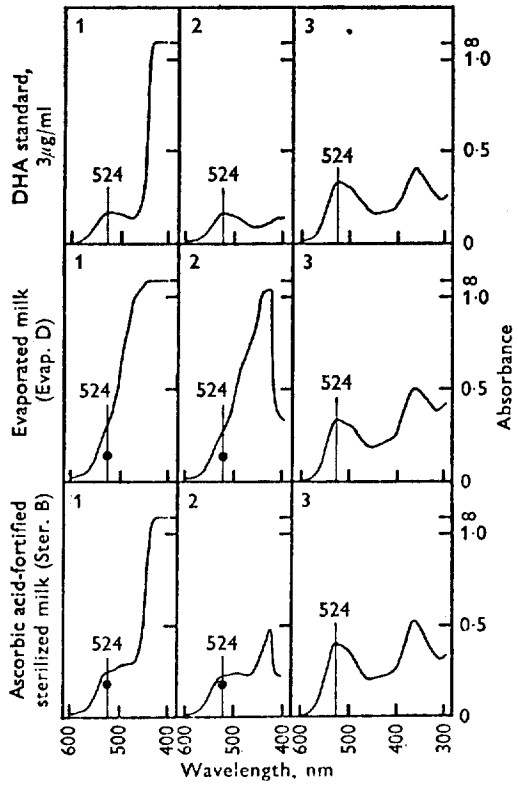

Fig. 1. The absorption spectra of the 2,4-dinitrophenylhydrazones of dehydroascorbic acid (DHA) and of the oxidized extracts (ascorbic acid (AA) + DHA + dioxogulonic acid + interfering substances) of evaporated and AA-fortified sterilized milks, 40 min after development of the colour with 85 % sulphuric acid. (1 and 2), before chromatography, 42 % sulphuric acid (1) or the corresponding blank (2) in the reference cell; (3), after chromatography, 42 % sulphuric acid in the reference cell. The correct absorbance due to AA content as calculated by the chromatographic procedure is shown (●). Because of the larger volumes of test solution used, the chromatographic procedure results in higher absorbance values.

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RESULTS

Absorption spectra of the hydrazones

For all the milks, the typical absorption curve of DHA with a peak at about 524 nm (Fig. 1) was obtained only after chromatography of the hydrazones.

Evaporated milks. Without chromatography, all the curves fell steeply in the 524 nm wavelength region, whether 42 % sulphuric acid solution or the corresponding blank was in the reference cell. Fig. 1 shows the absorption curves obtained for the oxidized extract of Evap. D before and after chromatography.

Sterilized milks. Before chromatography, the curves of the hydrazones of the untreated extracts showed a very small shoulder, but this was in the region of 500 nm. Those of the oxidized extracts indicated that DHA might be present, but there was also a component with a peak at about 495 nm which was not eliminated when the corresponding blank, instead of sulphuric acid solution, was in the reference cell. Fig. 1 shows the absorption curves obtained for the oxidized extract of Ster. B, before and after chromatography. The reduced extracts of these milks were not studied since only AA was being determined.

Table 1. *Mean values for vitamin C content (mg/100 ml) of reconstituted evaporated milks and of fortified sterilized milks,* determined by titration with 2,6-dichlorophenolindophenol (DCP) and from the absorbance values of the 2,4-dinitrophenylhydrazine (DNPH) derivatives at 524 and 540 nm*

	Ascorbic acid				Dehydroascorbic acid			
		DNPH method				DNPH method		
		No chromatography†		With chromatography‡		No chromatography†		With chromatography‡
Milk	DCP method	524 nm	540 nm	524 nm	DCP method	524 nm	540 nm	524 nm
Evaporated								
A	0·80	0·60	0·57	0·46	0·08	0·11	0·09	0·02
Ba	—	0·25	0·21	0·08	—	0·10	0·09	0·01
Bb	0·37	0·18	0·15	0·07	0·23	0·09	0·10	0·00
C	—	0·50 0·49‡	0·50 0·50‡	0·39	—	0·04 0·08‡	0·07 0·08‡	0·01
D	—	0·87	0·84	0·63	—	0·04	0·10	0·03
E	—	0·53	0·49	0·49	—	0·11	0·12	0·02
Fortified sterilized								
A	3·63	3·00	3·03	3·01	—	—	—	—
B	5·78	4·77	4·81	4·28	—	—	—	—

* A proprietary fortified and sterilized liquid baby food. Details given in text.
† Coupled at 37 °C for 3 h.
‡ Coupled at room temperature (about 20 °C) for about 19 h 30 min.

AA content

Table 1 shows that the DCP method gave higher AA values for the 2 evaporated milks studied (Evap. A, Bb), and for the sterilized milks (Ster. A, B), than were obtained by the DNPH methods.

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Without chromatography of the hydrazones and using the absorbance values at 524 nm, the AA content of the 5 evaporated milks ranged from 0·18 to 0·87 mg/100 ml. Slightly lower values for AA content were generally obtained using the absorbance values at 540 nm. Coupling with DNPH for 19½ h at room temperature, as was used in the chromatographic procedure, gave with Evap. C the same AA values as coupling for 3 h at 37 °C. The values of 3·0 and 4·8 mg AA/100 ml for Ster. A and B, respectively, indicated that even after 64 days of storage in the laboratory at ambient temperature and exposed to light, these samples apparently still contained a considerable amount of AA.

After chromatography of the hydrazones, the AA content of the evaporated milks was generally 0·08–0·21 mg/100 ml less than the values obtained from the absorbances determined at 540 nm without chromatography. The value for Evap. E remained unchanged, however. Whereas almost identical and very low values for the AA content of Evap. Ba and Bb were obtained by the chromatographic procedure, there was a discrepancy in the results obtained without the chromatography step on account of the high absorbance of the blank of the oxidized extract of Evap. Bb. This may have been due to the instability of the absorbance readings which is typical of strongly heated milks. Of the 2 sterilized milks, only the value for Ster. B showed any appreciable decrease after chromatography of the hydrazones, and the high level of AA in these milks was confirmed.

DHA content

Of the 2 milks analysed using the DCP method, the value of 0·08 mg DHA/100 ml for Evap. A was similar to that found by the DNPH method without chromatography, whilst that of 0·23 mg/100 ml for Evap. Bb was considerably higher.

Using the absorbances at 524 and 540 nm before chromatography of the hydrazones, the values found for the DHA content of the 5 evaporated milks were very low and ranged from 0·04 to 0·12 mg/100 ml. Using the chromatographic procedure virtually zero values were obtained.

DISCUSSION

The DCP method gave higher values for vitamin C content (AA + DHA) than were obtained by the DNPH methods. For Evap. Bb, which contained only a trace of vitamin C (0·07 mg/100 ml) as measured by the DNPH method with chromatography of the hydrazones, the DCP method gave a value of 0·60 mg/100 ml. The corresponding values for Evap. A were 0·48 and 0·88 mg/100 ml. Higher values for AA were obtained also in the sterilized milks by the DCP method.

In the DNPH method it was necessary, for both types of milk, to carry out the chromatographic separation of the hydrazones to ascertain that AA was present, although the error was small when this step was omitted. The AA contents of the various samples of evaporated milks differed, and after chromatography of the hydrazones the highest level, 0·63 mg/100 ml in Evap. D, was almost a third of the AA content found in freshly secreted cow's milk (Kon & Watson, 1936) whereas only a trace was present in Evap. B. It is likely that the AA remaining in the evaporated milks after processing is reasonably stable and that the tinned surface of the can may assist in this stability by the removal of oxygen, as reported by Kohman (1923) for canned

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fruit. The low level of DHA in the evaporated milks was consistent with the known heat lability of DHA (Hartman & Dryden, 1965). The high level of AA found in the fortified sterilized milks even after storage for a long period in the light could be accounted for by their low initial oxygen content (Ford, Porter, Thompson, Toothill & Edwards-Webb, 1969).

That the absorbance of other DNPH derivatives besides that of DHA was measured by the method of Roe *et al.* (1948) was evident from the shape of the absorption curves (Fig. 1) and also from the instability of the absorbance readings (Toothill *et al.* 1970). The determination of the absorbance values at 540 nm, as suggested by Mills & Roe (1947), rather than at the wavelength maximum of the DHA derivative, did not, however, overcome the effect of interfering substances. The inclusion of the chromatographic step generally resulted in lower values for AA and DHA and this decrease was due to the removal of interfering substances since mean recoveries of 97 and 100% were obtained when 0·5 mg AA was added to 100 ml of reconstituted milk (Evap. B). Unfortunately, the chromatographic procedure is very time-consuming, but for products containing low levels of DHA and DOA, such as evaporated milks, it could be shortened by using only the oxidized extracts, which gives a combined value for AA + DHA + DOA. For the present series of evaporated milks this procedure would lead to a maximum error of 0·05 mg/100 ml reconstituted milk compared with a maximum error of 0·21 (Evap. D) if chromatography were omitted. For Ster. A and B, the error would be 0·27 and 0·42 mg/100 ml, respectively.

An isomer of AA, namely D-isoascorbic acid (IAA) also known as erythorbic or D-araboascorbic acid, is allowed in some countries, but not in the United Kingdom, as an antioxidant in foods. It has only about one-twentieth the antiscorbutic activity of AA (Smith, 1946). IAA has been reported to prevent the loss of AA in fruit juices (Esselen, Powers & Woodward, 1945) and to reduce the headspace oxygen in cans of apples (Hope, 1961). We have found that the chromatographic procedure used in the present work fails to separate the osazone of IAA from that of the naturally occurring form of AA and, in the unlikely event of IAA being added to milk, the DNPH method would require modification. Methods are available for separating the less stable free acids (Weeks & Deutsch, 1967).

The present work shows that of the 3 methods used for the determination of vitamin C in evaporated and fortified sterilized milk, that involving the chromatographic separation of the DNPH derivatives was the most specific.

We are grateful to Mr J. Edwards-Webb for the vitamin C determinations using DCP.

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INTRODUCTION.

CABBAGE (*Brassica oleracea* L. var. *capitata* L.), is regarded as one of the most important green leafy vegetables in our diet. This is on account of the fact that this vegetable is palatable, obtainable over long periods of the year, and rich in certain vitamins and minerals.

The nutritive value of cabbage varies with variety, season and cultural practices, and also with soil nature and type, fertilizers and irrigation.

Burrell *et al.* (1940) reported that the variety "All Season", grown on an unfertilized clay loam, contained 72·6 mg. ascorbic acid per 100 grs. of fresh material, while the same variety grown on a fertilized clay loam contained 89·4 mg. per 100 grs. This was also true for other cabbage varieties. In this study the effect of *kraal manure* on ascorbic acid values in cabbage is discussed.

The wide variations in the composition of the different varieties demand that discretion be used in the selection of a variety for processing or for fresh consumption. This applies especially to conditions where demands are made on the nutritive characteristics of this vegetable.

Analysis of the whole head (Table I), made by Richardson, at different st ages of development, show no striking differences in the composition after the early stages. These determinations were made on water-free material.

TABLE I.—*Composition of Cabbage During Development.* (Richardson)

Whole head.	H°O.	Protein.	Fat.	Fibre.	Ash.
	%	%	%	%	%
June 2.....	88·39	2·71	0·67	0·94	3·43
June 26.....	87·93	2·94	0·69	1·32	2·55
July 3.....	86·01	2·62	0·74	1·55	2·70
July 10.....	87·46	2·72	0·54	1·51	2·08

Average values of the compositions of cabbage in general are also given by other workers:—

TABLE II.—*Composition of cabbage.*

	H_2O.	Protein.	Fat.	Carbohydr. excl. Fibre.	Fibre.	Calories per pound.	Ash.	Ca.	P.	K.	Fe.
	%	%	%	%	%		%				
Fox and Golberg (1944)	92·4	1·4	0·2	4·3	1·0	130	0·75	45 mg.	29 mg.	—	1·1 mg.
Russell (1926)....	—	—	—	—	—	—	—	78 mg.	64 mg.	438	1·4 mg.

The values given by Fox and Golberg (1944)) are calculated on fresh material, and give an average for all varieties.

The nitrogen distribution, as determined by Yoshimura (1910), in fresh cabbage leaves containing 89·4 per cent. H_2O and 0·311

per cent. of nitrogen, was as follows:— protein nitrogen, 0·091 per cent.; ammonia nitrogen, only traces; non-protein nitrogen, including phosphotunstic precipitate, 0·051 per cent.; other forms 0·169 per cent. In fresh material he also found histidine; arginine, 0·7 grs.; lysine, 0·2 grs.; choline, 0·3 grs.; and betaine, 0·1 grs.

According to Ozaki (1930), the saturated acids, constituting 16·3 per cent. of the total acids, contained arachidic 10 per cent., and at least 80 per cent. palmitic acid, together with a small amount of myristic, but no stearic; the unsaturated acids contained linolenic 10 per cent., an isomer of linolic 75 per cent., and an isomer of oleic, at least 10 per cent.

An ether extract was prepared by Chibnall and Channon, (1929) by expressing the juice of the leaves, free from thick midribs, coagulating at 70° C., cooling, filtering, removing the excess liquid by pressure, drying the coagulum and extracting with ether. The following amounts of the constituents, expressed as percentages of the extract, are given in the final report of the series:—

Chlorophyll (a and b), 9·3; carotene, 0·5; xanthophyll, 0·8; calcium phosphatidate, 18·5; unidentified calcium salts, 5·1; identified iron compound, 3·0; glycerides and waxes, 17·4; glycerol, 1·3; unsaponifiable matter, 12·3; sterols by digestion, 4·4; other unidentified substances, 13·3. The sugar content varies from 3 to 4·3 per cent. in fresh cabbage.

Methods and Materials.

The principle object of this study was to determine the effect of variety, season and fertilization on the absorbic acid content of cabbage.

Ascorbic acid was determined by making use of the strong reducing action of the vitamin on 2, 6-dichlorophenolindophenol solution (Bessey and King, 1933), and employing the modifications and sampling as described by le Riche (1945) for lettuce and other vegetables.

In this study special attention was given to the sampling technique for ascorbic acid determinations, as well as to the sample size for cabbage analysis. In biological material, and especially plant material, rich in enzymes, the validity of analytical results depends upon the sampling accuracy from the field plots and in the laboratory speed of analytical procedure and the accurancy of the methods employed for the specific determinations on the material analysed.

Should the sample size, from the field plots, have been determined, and the small amounts taken from these again, in the laboratory, be minced, shredded or cut into too small pieces, the degree of the liberation of enzymes in the cell sap will be too great, and the vitamin may be destroyed, or partly oxidized, before the extraction acid, metaphosphoric acid—can penetrate sufficiently to inactivate the oxidizing enzymes. In this study 20-gramme samples were taken from a number of heads (described below), the green and white leaves being treated separately. These 20-gr. samples were taken by cutting small pieces, with a stainless steel knife, from the white and green leaves of a number of heads respectively, and immediately weighing into a 3 per cent. metaphosphoric acid solution. The samples were ground under the acid solution with the aid of clean quartz sand until a homogeneous pulp was obtained. This was

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then centrifuged and the clear supernatant liquid used for the final determination.

Since only a portion of the cabbage is used for each assay, the accuracy of sampling must be determined. As will be seen later on, the concentration (Table V) of vitamin C may vary in the different parts of the cabbage.

The accuracy of the sampling method was ascertained by the analysis of at least four samples from each cabbage or group within the sample taken from the field plot, and determining the coefficient of variation of a single assay result. The average results thus obtained from about one hundred samples are summarized in Table IV.

In addition to the determination of the accuracy of the analyses, the most favourable sample size, i.e. number of heads per sample, was determined. This was necessary, as it was found that there are variations in ascorbic acid content from head to head within a variety. For this determination one variety was used and analyses were conducted on samples containing one to four heads, each of which were replicated four times, and four ascorbic acid determinations made on each sample. Twenty-gramme samples were taken and ascorbic acid determination made.

TABLE III.—*Determination of sample size for the estimation of ascorbic acid in cabbage.*

Number of heads per sample.	Repetitions.	Coef. of variation.
1	15	9·5
2	10	7·7
3	10	2·8
4	10	2·1

TABLE IV.—*Sampling errors in vitamin C. estimations on cabbage varieties.*

Part of cabbage.	Coefficient of variation for the estimation of vitamin C. in a single sample.
Green leaves (outer)	5·6
White leaves (inner)	5·4
Midribs	4·7

Very little information is available regarding the sampling error, when estimating vitamin C. Olliver (1938) quotes results on duplicate samples of strawberries, giving coefficients of variation of 6·1 for unripe and 2·9 for ripe red fruit. Wokes and Organ (1943), who worked on ascorbic acid in tomatoes, reported coefficients of variation for ascorbic acid determinations of 5·8, 3·1 and 8·2 for whole fruits, the skin, and the flesh and juice, respectively. Le Riche (1945), reported that the coefficient of variation for ascorbic acid determinations on lettuce was 4·64. The abovementioned results are of the same order as those for cabbage, as given in Table IV.

In addition to the determination of sampling errors and sample size, actual titration errors in the final determination of ascorbic

acid were also determined. For this, at least four titrations were made on each extraction and the end-points usually checked by two observers. From the results of a hundred titrations, it was determined that the titration error, on cabbage samples, was 1·57 per cent. Titrations were relatively easy, as the liquid to be titrated is colourless and clear, and end-points distinct.

In the ascorbic acid analysis the accidental errors may be classified into different groups:—

(1) Sample-size errors;
(2) errors due to cutting from the different heads, pulping and standing, etc.; and
(3) titration errors.

If the coefficients of variation resulting from these causes are called V_1, V_4, V_3, etc., then the total coefficient of variation will be:—

$$V = (V_1^2 + V_4^2 + V_3^2)^{\frac{1}{2}}$$

In the study the sample-size error (V_1), titration error (V_3), and the total errors in a single sample were determined. The error for a single sample, (V_4) (Table IV), minus the titration error (V_3), gives the calculated error due to cutting from the different heads, pulping, standing, etc. If this is represented by V_2, then;

$$V_2 = (V_4^2 - V_0^2)^{\frac{1}{2}}$$

therefore, V_2 for green leaves, white leaves and midribs, is 5·49, 5·27 and 4·46, respectively.

The total errors for the determination of ascorbic acid in cabbage are:—

$$V = (V_1^2 + V_4^2)^2$$

V for green leaves = 6·10
V for white leaves = 5·91
V for midribs = 5·21

Significant differences in vitamin C. concentration were found between different varieties of cabbage, which is in accord with the work of Lampit, Baker and Parkinson (1945).

The most important factor, however, in studying the composition of cabbage is the accuracy of sampling. The above workers experienced similar difficulties. They used only one plant in their samples and the coefficients of variability of their results vary from 10·3 per cent. to 23·8 per cent., while in this study the variability, calculated from the errors due to sample size and the sampling, as discussed, are 6·1 per cent. for the green outer leaves, 5·9 per cent. for the inner white leaves and 5·2 per cent. for the midribs. On account of the variations in the concentration of ascorbic acid in the different leaves of cabbage, it was necessary to analyse the parts separately. If this is not done, the errors are still larger than reported. The varieties analysed by the above authors, give values of the same size order as reported in this study, but the total errors are much smaller on account of the fact that the samples from the field were much larger, and thus more representative of the whole.

From the above it is evident that the number of heads from which composite samples are taken, is a very important factor and

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that under no circumstances can one head of a variety be used in order to obtain significant results; more than one extraction is also necessary.

Enzymes.

Oxidizing enzymes occur in the highest concentration in the outer green leaves, while these are also very active in the inner white leaves. In the sampling and processing of cabbage, great care should be taken to conserve ascorbic acid and to eliminate excessive disruption of the cells, which will liberate the oxidizing enzymes. When cabbage is shredded into 0·25-inch shreds, up to 40 per cent. of the ascorbic acid is destroyed within ten minutes. Thus, in the processing of this vegetable, rough handling, bruising, over-blanching, leaching and excessive temperatures will destroy the quality of the final product.

The losses sustained in ascorbic acid during cooking are as high as 50 per cent., while stewed and baked cabbage loses up to 85 per cent. The smallest losses are obtained by adding whole cabbage leaves, with enzemes limited to the cells, in small portions to boiling water, so that the heating is rapid and enzymes consequently rapidly inactivated. Nearly all the vitamin C. can be retained in this manner.

Pyke (1942) reported that grating of cabbage resulted in a loss of 34 per cent. of the ascorbic acid within five minutes. He found that shredding with a sharp knife produced no significant loss of the vitamin, although bad, ineffective cutting and bruising caused about 10 per cent. loss. These losses are lower than was found to be the case in this study.

For the above reasons, the sampling of cabbage for ascorbic acid assays were carried out by cutting small pieces from the heads with a stainless steel knife, thus avoiding excessive cell damage. Shredding or mincing of cabbage, prior to sampling, should not be resorted to under any circumstances.

Results.

Ascorbic Acid in Different Parts of Cabbage Heads.

The concentration of ascorbic acid in the different parts of cabbage heads was determined by making triplicate analyses on each leaf and midrib of three heads of cabbage. The leaves were carefully removed, beginning from the surface of the heads, and separate analysis made on the leafy and midrib parts. The mean values of the analyses on each leaf are given in Table V. On the basis of the experiment, these are believed to be free from errors resulting from oxidase destruction of the ascorbic acid during the preparation and sampling of the material.

TABLE V.—*Distribution of ascorbic acid in cabbage. (Variety: Private stock).* (as mg/100 grs.)

Leaf number (taken from the outside).	Vitmanin C in soft tissue.	Vitamin C. in midribs.
1	87·8	—
2	79·9	—
3	90·6	90·27
4	89·7	90·27
5	105·6	90·58
6	88·1	90·6
7	94·9	86·6
8	94·9	73·4
9	73·4	70·4
10	70·4	65·8
11	58·1	66·4
12	63·3	66·7
13	61·2	65·2
14	—	66·4
15	67·3	70·9
16	62·7	62·7
17	65·2	67·3
18	68·9	68·9
19	64·3	67·3
20	74·4	74·1
21	69·5	67·3
22	65·8	65·3
23	65·0	74·9
24	65·2	65·5
25	70·4	70·7
26	74·7	71·6
27, 28, 29	73·7	73·2
30, 31, 32, 33	78·6	75·2

From Table V it appears that, for the variety analysed, there was a slight decrease in ascorbic acid from the ninth leaf. The difference between the green and white leaves is significant, but not as large as usually reported. In all cases the concentration in the midrib was more or less equal to that of the leafy tissue.

Werder and Antener (1938) reported that green cabbage contained 42·7 mg. per 100 grs., white cabbage contained 24·5 mg. ascorbic acid per 100 grs. These figures are for a certain variety; and he reports that the difference may be larger for some other varieties. The fact that the white inner leaves and the stalk are rich in ascorbic acid, indicates that the vitamin is not only synthesized in green or chlorophyll-containing leaves, but also in the white tisues lacking chlorophyll.

THE EFFECT OF VARIETY ON ASCORBIC ACID IN CABBAGE.

In all vegetables, varietal differences are the most important factors affecting the final chemical composition of the variety. Cabbage varieties differ in growth, shape of heads and leaves, and percentage of leaves exposed to the sun, resulting in chlorophyll development and variations in chemical composition. These factors, together with genetic characteristics, all influence the ascorbic acid concentration to smaller or larger degree. Table VI gives the ascorbic acid values of green leaves and white leaves, as well as the moisture content of each variety. The results are calculated from five replications on each variety.

TABLE VI.—*Ascorbic acid content of cabbage varieties.*
(In mgr. per 100 gr. fresh material).

Variety.	Green Leaves.			White Leaves.		
	Mean.	Std. Dev.	H_2O.	Mean.	Std. Dev.	H_2O.
Main Crop	75·3	4·64	92·68	74·1	2·20	92·09
Jersey Wakefield	67·0	3·49	92·70	74·8	4·24	92·10
Mammoth	69·5	4·47	92·59	75·3	3·56	91·95
Copenhagen Market	66·7	0·60	93·07	72·8	3·10	92·30
Private Stock	53·9	1·65	92·19	59·4	3·76	92·67
Golden Acre	54·5	1·63	93·28	64·7	2·12	92·94
All Season	112·3	4·23	—	62·8	7·52	—
Prize Drumhead	108·0	11·54	—	44·2	5·54	—
Summer Spits	81·2	2·35	—	57·0	1·04	—
Giant Drumhead	74·1	3·00	—	51·7	3·68	—
Charleston Wakefield	79·7	1·64	—	87·1	1·82	—
Oxheart	79·3	5·36	—	53·6	3·61	—
Succession	114·3	5·01	—	62·2	2·05	—
Glory of Enkhuizen	101·0	4·09	—	37·8	4·21	—
Savoy Drumhead	88·0	3·91	—	73·2	5·92	—
Large Oxheart	126·0	4·41	—	88·5	4·32	—
Large Brunswick	100·3	4·03	—	48·7	3·91	—
All Head	64·8	2·12	—	48·4	3·18	—
Early Flat Dutch	62·9	2·30	—	51·1	4·00	—
Brunswick Drumhead	141·6	6·10	—	38·9	4·00	—
Late Flat Dutch	107·0	7·39	—	71·0	4·87	—
American Sure Head	92·3	6·32	—	51·0	4·19	—
Cape Spits	104·0	6·29	—	36·0	3·14	—
Early Round Dutch	95·7	6·31	—	48·6	4·01	—
Mammoth Red Rock	84·6	4·91	—	64·3	5·32	—
Early Market	60·8	3·43	92·92	60·49	1·85	91·86

From Table VI it is evident that in cabbage the ascorbic acid values vary from variety to variety, and in the green leaves and white leaves.

Much work has been conducted on varietal variations in fruit and vegetables, and Hamner and Maynard (1942) recently reviewed all the studies dealing with this topic. They concluded that differences between specific varieties are not necessarily the same in all areas, and that external factors, which will be discussed below, may influence chemical composition and growth. It seems justifiable (Maynard and Beason, 1943) to conclude, however, that the accumulation of ascorbic acid in plants is a characteristic of species and variety, and that this genetic influence may overwhelm external factors. In leafy vegetables, however, light, exposure to the sun and rate of growth are important for the accumulation of ascorbic acid.

SEASONAL VARIETIES IN ASCORBIC ACID.

It is commonly recognized that the ascorbic acid content of certainvegetables and fruit, varies with the locality in which they are grown. This has been attributed to differences in soil, available nutrients, water-holding capacity of the soils, exposure to light and to sun, and altitude (Beason 1941).

In the studies conducted on cabbage varieties over a period of three seasons, ascorbic acid differences within the varieties were observed (Table VII).

TABLE VII.—*Seasonal variations in ascorbic acid concentration of cabbage varieties.*

Variety.	Season 1944.		Season 1945.	
	Green.	White.	Green.	White.
Copenhagen Market.....	84·2	41·1	54·2	65·4
Private Stock.....	90·8	43·5	61·9	63·9
Jersey Wakefield.....	99·2	70·1	53·4	64·5
Danish Ball.....	74·7	50·5	55·1	63·1
Summer Spits.....	101·1	56·7	110·8	82·1
Main Crop.....	87·4	45·2	57·0	66·4
Golden Acre.....	62·6	40·0	55·1	54·1
American Sure Head....	92·3	50·0	81·9	50·8
Giant Drumhead.....	51·0	27·2	87·4	60·2
All Season.....	68·6	50·4	125·5	70·1
MEAN VALUES....	81·19	47·57	62·25	64·06

The seasonal variations, as indicated in Table VII, are evident and interesting in that the ascorbic acid values of the green leaves during the 1944 season much higher than in the following season, as can be observed from the mean values for all the varieties. During the 1945 season the variation in ascorbic acid concentration between green outer and white inner leaves is very small, while in the previous season the opposite was true. The actual reasons for this variation are difficult to assess, as ascorbic acid may be influenced by many factors. The most important recorded difference between the two seasons was that the 1945 season was very dry and hot, which resulted in slow and very difficult growth.

Influence of Manurial Treatments on Ascorbic Acid in Cabbage Varieties.

In this trial the influence of *kraal manure* on ascorbic acid concentration in cabbage was determined. The plots were in a planned plant nutrient experiment, and sufficient plot replications were made to give significant results. Cabbage samples for each variety were taken from all the plots, and the necessary analyses made. The treatments were 20 and 40 tons of kraal manure, respectively, per morgen. The plots were in a randomized field trial, and all other conditions for growth were similar. The plants grew under very adverse climatic conditions, as during their final growth period they were subjects to severe drought conditions.

TABLE VIII.—*Influence of two levels of kraal manure on ascorbic acid in cabbage varieties as mg/100 grs.*

Variety.	29 TONS/MORGEN.		40 TONS/MORGEN.	
	Green.	White.	Green.	White.
Early Market	54·7± 2·49	59·86±3·5	60·8± 3·43	60·49±1·85
Main Crop	56·9± 3·78	66·44±4·8	75·4± 4·64	74·10±2·20
Mammoth	56·8± 1·38	57·20±2·2	69·5± 4·47	75·4 ±3·56
Copenhagen Market	54·2± 1·93	65·42±2·9	66·7± 0·60	72·8±3·10
Golden Acre	55·1± 2·55	54·1±0·54	54·5± 1·65	64·8±2·12
Danish Ball	55·0± 1·18	63·1±2·06	55·5± 1·60	62·7±0·94
Jersey Wakefield	53·4± 4·12	64·6±7·14	67·1± 3·49	74·9 ±4·24
American Sure Head	81·9± 1·89	50·8±4·41	104·1± 9·34	57·6 ±4·81
Prize Drumhead	67·3± 2·31	48·6±1·97	108·1±11·54	44·3±5·54
All Head	88·4± 6·67	67·6±2·80	92·9± 4·22	61·5±5·89
Oxheart	74·2± 7·92	60·5±4·01	79·3± 5·36	53·6±3·61
Early Drumhead	105·3±11·10	57·9±5·01	108·9± 5·76	54·6±3·02
Succession	87·3± 4·21	55·2±3·29	114·4± 5·01	62·3±2·05
Early Stone Head	83·9± 5·03	61·9±5·35	109·8± 3·69	67·6±2·09
Private Stock	61·9± 3·59	63·9±4·39	54·0± 1·65	59·4± 3·76
All Season	125·5± 5·76	70·1±5·75	112·3± 4·23	62·8±7·52
Summer Spits	110·8± 2·87	82·1±5·05	81·2± 2·35	57·0±1·04
Giant Drumhead	87·4± 8·03	60·2±0·65	74·2± 3·02	57·7±3·68

The above results indicate that the ascorbic acid concentration is higher in the cabbage varieties which received 40 tons per morgen. This applies to all the varieties except the last four in the table, viz. "Private Stock", "All Season", "Summer Spits" and "Giant Drumhead", which showed a higher concentration for the varieties grown with 20 tons kraal manure per morgen.

The contradictory results obtained by reputable investigators in different parts of the world on the effect of fertilization on the ascorbic acid content of food plants might justifiably lead to some doubt as to whether mineral nutrition of the plant does influence its ascorbic acid content.

Potter and Overholser (1933) concluded that apples from trees receiving applications of a complete fetilizer were a better source of vitamin C. than those from trees not so fertilized.

Todhunter (1939) on the other hand, reported that Winesap apples did not show different amounts of ascorbic acid as a result of fertilization. A relationship between Vitamin C. and the amount of nitrogen in apples was first suggested by the observations of Bracewell, Hoyle and Zilva (1930), who found marked and consistent differences in ascorbic acid content between two varieties that were normally different in their nitrogen content. Further studies on the effect of fertilization on the ascorbic acid content of fruit and vegetables were made by Kessler (1939); Roy and Bahrt (1940), who reported on the effect of magnesium and zinc; Hamner, Lyon and Hamner (1942); Nelson and Mottern (1932); and Hester (1941. The results of these investigators are contradictory in nature and no positive conclusions can be drawn.

The treatments in this experiment show significant variations which may be ascribed to an increase in the plant nutrients and organic matter, or to the more favourable retention of moisture in

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the soil. The soil is a sand, and organic matter will undoubtedly affect this factor. In the 40-ton treatment, not only the minerals are increased, but, what is of more importance, according to the work of Bracewell *et al.* the organic nitrogen content of the soil is also doubled.

Further experiments are being conducted on the effect of inorganic and organic fertilizers on the ascorbic acid content of cabbage varieties.

SUMMARY.

1. A survey has been made of the chemical constitution of cabbage.

2. The sample size, sampling errors and titration errors for cabbage have been determined.

3. Oxidizing enzymes are more concentrated in the green outer leaves, and very active in the white inner leaves. Shredding, mincing or any process whereby the enzymes are liberated, results in the destruction of ascorbic acid. Ascorbic acid losses during the cooking of cabbage may be as high as 50 per cent., and up to 80 per cent. during baking.

4. Green leaves are richer in ascorbic acid than the inner white leaves, but differences for all the leaves are not very large. Midribs contain as much ascorbic acid as the leafy tissue.

5. Ascorbic acid varies with variety and is influenced by season and applications of kraal manure. Twenty-six varieties have been studied.

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Biochem. J. 30, 2291-8 (1936)

CCCXVIII. ON THE AMOUNT OF ASCORBIC ACID IN BLOOD AND URINE. THE DAILY HUMAN REQUIREMENTS FOR ASCORBIC ACID.

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(*Received 17 September 1936.*)

THE investigations described in this contribution were undertaken [van Eekelen, 1936] to answer the following questions: (1) Do the ascorbic acid content of the blood and the daily urinary output of it depend on the amount of vitamin C stored in the human organism? Is it possible to get an insight into the state of saturation of a given subject, for the special purpose of detecting hypovitaminosis, by determining the ascorbic acid in blood or in urine? (2) What are the normal daily requirements for ascorbic acid of human adults?

Van Eekelen *et al.* [1933, 1] and Johnson & Zilva [1934] have shown that, following intake of large doses of ascorbic acid, the organism can be saturated to such an extent that a further dose of ascorbic acid is partly or entirely excreted in the urine. In this way the storage of ascorbic acid in a certain subject can be investigated by determining the amount of ascorbic acid required to reach the point of saturation. We shall consider this point to be reached when a daily dose of 250–400 mg. ascorbic acid results in a definite rise of urinary output (about 30 mg. above the normal average). This does not indicate complete saturation, in which case nearly the total amount taken in is excreted.

The questions arise: What is the amount of ascorbic acid in the blood when this point of saturation has been reached? Do the amount in the blood and the daily urinary output of ascorbic acid decrease when a saturated experimental person lives for some time on a diet devoid of vitamin C? When a decrease results an increase can be expected by saturating the person again.

By this experiment it is possible also to calculate the daily requirements for the vitamin supposing that the quantity of ascorbic acid necessary to saturate the subject at the end of the dietetic period equals the amount required during this period. No reasons against this supposition could be detected. To be sure that the dietetic prescriptions were followed as strictly as possible and that the urinary examinations could be done in time the writer himself was the experimental subject.

METHODS.

Determination of ascorbic acid in urine. The ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol standardized against crystalline ascorbic acid (about 10 ml. indicator corresponds to 1 mg. ascorbic acid). Urine contains besides ascorbic acid other substances reducing the indicator, e.g. ergothioneine [Sullivan & Hess, 1933] and thiosulphates [van Eekelen, 1934]. For this reason it is necessary to remove these interfering substances by precipitation with mercuric acetate [Emmerie, 1934; Emmerie & van Eekelen, 1934; vau Eekelen & Emmerie, 1936]. The urine is examined immediately after it has been voided.

Determination of ascorbic acid in blood. In blood also reducing substances other than ascorbic acid are present (e.g. ergothioneine, glutathione) which can be removed likewise by precipitation with mercuric acetate. Thus, the blood being precipitated with trichloroacetic acid, mercuric acetate is added, the mixture neutralized with $CaCO_3$ until slightly acid (Congo red paper remaining unaltered, blue litmus paper turning to red) and treated with H_2S [Emmerie & van Eekelen, 1934].

Hardly any reducing substances are present when the blood filtrate is titrated after precipitation with trichloroacetic acid (before the treatment with mercuric acetate and H_2S) even when pure ascorbic acid has been added previously. From this experiment we have earlier concluded [van Eekelen *et al.* 1933, 2] that the ascorbic acid in the blood is present in the reversibly oxidized state and that an added quantity of ascorbic acid will be reversibly oxidized by the blood. The following determinations however demonstrate in agreement with Kellie & Zilva [1935; 1936] that this supposition is not true [van Eekelen, 1935].

Table I. *mg. ascorbic acid per 10 ml. ox blood, plasma and erythrocytes.*

	After precipitation with CCl_3COOH	After reduction with H_2S	After precipitation with CCl_3COOH and $Hg(CH_3COO)_2$ and reduction with H_2S
Blood	0	0·28	0·042
Plasma	0·035	—	0·034
Erythrocytes	0	—	0·050
Blood + 0·9 mg. ascorbic acid	0·15	—	0·93
Blood, centrifuged; in 5 ml. plasma	0·75	—	0·75
Blood + 4·92 mg. ascorbic acid	3·31	—	4·91*
Plasma + 4·92 mg. ascorbic acid	4·89	—	4·91†

* Calculated 4·95. † Calculated 4·94.

Table I shows: (1) Ascorbic acid is present both in the plasma and in the erythrocytes. (2) Precipitation with mercuric acetate is necessary because after reduction with H_2S without precipitation with mercuric acetate a larger amount of reducing substances is found. This demonstrates the presence of reducing substances other than ascorbic acid. (3) In plasma ascorbic acid is present in the reduced state. (4) During the precipitation with trichloroacetic acid, the ascorbic acid becomes reversibly oxidized if erythrocytes are present. This oxidation occurs especially when the blood is oxygenated. After treatment with CO_2 or CO only a small part of the ascorbic acid is oxidized (Table II).

Table II.

	After precipitation with CCl_3COOH	After precipitation with CCl_3COOH and $Hg(CH_3COO)_2$ and reduction with H_2S
10 ml. aerated blood + 4·92 mg. ascorbic acid	3·31	4·91
10 ml. blood saturated with CO_2 + 4·92 mg. ascorbic acid	4·72	4·89
10 ml. blood saturated with CO + 4·92 mg. ascorbic acid	4·72	4·89

Thus we have offered sufficient evidence that the determination of ascorbic acid in blood is unreliable without treatment of the trichloroacetic extracts with mercuric acetate and H_2S. From the data above results the possibility of determining the vitamin in serum directly, without treatment with mercuric

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acetate and H_2S [Gabbe, 1934; Farmer & Abt, 1935; Abt *et al.*, 1936; Taylor *et al.*, 1936]. However, it is advisable to apply the mercuric acetate and H_2S procedure because in serum also other interfering reducing substances may occur or the ascorbic acid may be partly reversibly oxidized (e.g. after haemolysis).

Experimental details. The experimental person was a man, aged 30 years, height 178 cm., weight 90 kg. The investigations were done from August to December 1935. The diet devoid of vitamin C contained bread, butter, cheese, eggs and water. For the saturation tests crystalline ascorbic acid (Hoffmann-La Roche) was taken.

The course of the experiment is given in Table III and Fig. 1.

Table III.

Date (1935)	mg. ascorbic acid taken in	ml. urine excreted	Daily urinary output mg. ascorbic acid	mg. ascorbic acid per l. blood	g. faeces excreted per day	mg. ascorbic acid in faeces	Number of petechiae. Left arm (*L*), right arm (*R*)
29. vii	—	—	—	—	—	—	—
30	—	742	13·1	—	—	—	—
31	—	875	12·4	—	—	—	—
1. viii	—	833	13·4	11·2	175	4·6	—
2	250	929	16·6	11·7	—	—	—
3	250	780	23	16·8	120	3·6	—
4	—	793	17·2	15	140	3·8	—
5	250	769	*45·6*	17	143	4·3	—
6	—	675	15·8	15	140	4·1	—
7	—	754	13·7	—	76	3·2	—
8	—	—	—	—	128	3·3	—
13	—	—	—	9·6	—	—	—
17	—	1046	10·5	—	—	—	—
18	—	903	12	—	—	—	—
19	—	770	10·2	—	—	—	—
21	—	—	—	6	—	—	—
22	—	847	11·9	—	—	—	—
23	—	819	11·2	—	—	—	—
28	—	902	10·2	—	—	—	—
29	—	830	10·1	—	—	—	—
30	—	—	—	4·3	—	—	—
4. ix	—	—	—	—	—	—	*L* 0
7	—	910	9·2	—	—	—	—
8	—	845	8·4	—	143	4·2	—
9	—	—	—	3·7	—	—	—
13	—	855	9·1	—	—	—	—
14	—	777	8	—	—	—	*L* 0
15	—	790	7·6	—	—	—	—
16	—	—	—	3·1	—	—	—
23	—	713	9	2·5	—	—	—
24	—	807	8·1	—	—	—	—
28	—	889	8	—	—	—	*L* 2, *R* 1
29	—	—	—	—	142	4·7	—
1. x	—	—	—	3	—	—	—
4	—	727	6·7	—	—	—	—
9	—	769	7·8	—	—	—	—
11	—	—	—	2·3	—	—	—
12	—	—	—	—	—	—	*L* 3, *R* 2
21	—	660	7·2	2·2	—	—	—
26	—	—	—	—	—	—	*R* 1
28	—	789	7	1·9	145	7·4	—
29	2 × 250	678	8·8	—	—	—	—
30	2 × 250	764	9·1	—	—	—	—
31	2 × 250	699	10·9	5	—	—	—

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Table III (*cont.*).

Date (1935)	mg. ascorbic acid taken in	ml. urine excreted	Daily urinary output mg. ascorbic acid	mg. ascorbic acid per l. blood	g. faeces excreted per day	mg. ascorbic acid in faeces	Number of petechiae. Left arm (*L*), right arm (*R*)
1. xi	2 × 250	688	15·1	—	186	8·7	—
2	250	682	15·9	8·9	—	—	—
3	250	688	12·9	—	—	—	—
4	—	678	12·2	9·4	—	—	—
5	250	656	14·5	—	—	—	—
6	250	727	19	—	—	—	—
7	250	646	*35·5*	13·3	—	—	—
8	—	594	15	14·4	—	—	—
9	—	761	14·4	—	—	—	—
11	—	556	12·9	—	—	—	—
12	—	—	—	10·6	—	—	—
16	—	614	10·5	8·3	—	—	—
21	—	—	—	6·6	—	—	—
26	—	625	10·1	—	—	—	—
27	—	607	9·2	5·1	—	—	—
28	250	661	9·5	—	—	—	—
29	250	728	10·3	—	—	—	—
30	250	651	10·7	—	—	—	—
1. xii	250	683	13·3	—	—	—	—
2	250	737	15·8	—	—	—	—
3	250	604	21·6	—	—	—	—
4	250	556	*39·5*	13·2	—	—	—

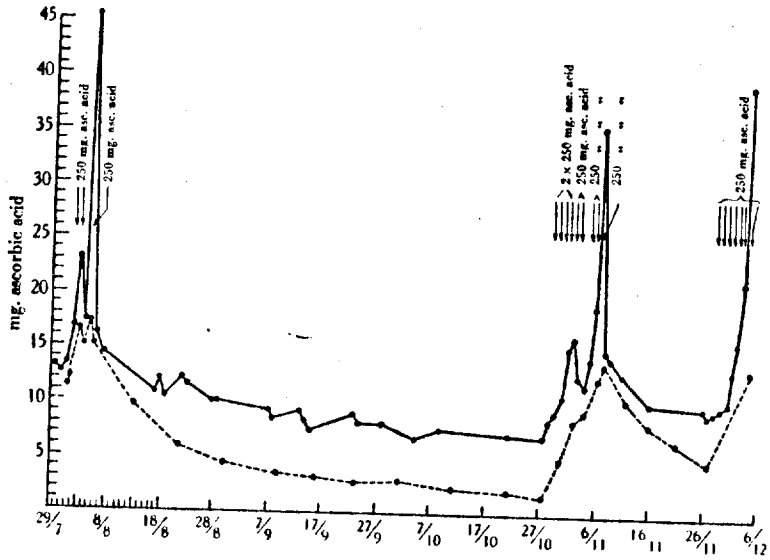

Fig. 1. •—• mg. ascorbic acid, daily urinary output.
•– – –• mg. ascorbic acid per l. blood.

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From this table it will be seen that the diet deprived of vitamin C began on 29 July. On the next 3 days the amounts of ascorbic acid excreted in the urine were determined (about 13 mg. per day). After an intake of 250 mg. daily during 3 days a definite rise in the urinary output was observed (a surplus excretion of about 30 mg. on 5 August).

By determining the ascorbic acid content of the faeces it was shown that an increased excretion with the faeces did not occur when large doses of ascorbic acid were given (see Table III).

Following this saturation the diet devoid of vitamin C was continued until 29 October. At intervals the ascorbic acid content of the blood and the daily urinary output were determined. The amount in the blood being 11·2 mg./l. before the experiment began was 11·7 mg./l. after 250 mg. had been taken and increased to 16·8 mg. after the second dose of 250 mg. (determination 3 hours after intake of ascorbic acid). On the day of this increased amount in the blood a somewhat larger urinary excretion was observed (23 mg. instead of 13 mg.). The next day (4 August) no ascorbic acid was taken in; the amount in the blood decreased to 15 mg./l. On 5 August 250 mg. were taken in again and a greatly increased excretion (45·6 mg.) resulted. Compared with the amount on 3 August the content of the blood only rose to 17 mg./l. (3·5 hours after intake). This increase was transitory, the next day 15 mg. were found. So it appeared that a surplus excretion of ascorbic acid in the urine occurs when a certain level in the blood (about 15 mg./l.) is surpassed. In the course of the dietetic period the daily urinary output slowly decreases; after 83 days from about 13 mg. to about 7 mg. with, however, large fluctuations. Compared with this observation the decrease of the amount in the blood is more important however only during the first period of the experiment (after 25 days from 15 mg. to 4·3 mg./l.). During the next period the decrease was less marked (after 59 days from 4·3 to 1·9 mg./l.).

After 84 days of the regimen ascorbic acid was again taken. To reach the point of saturation more quickly two doses of 250 mg. were taken daily during the first days, then daily doses of 250 mg. were taken (as at the beginning of the experiment) to avoid the point of saturation being suddenly largely exceeded. 3250 mg. were necessary to restore saturation (7 November, 35·5 mg. were excreted in urine). In the course of the saturation the amount in the blood and in the urine increased. At the point of saturation the amount in the blood was 14·4 mg./l. The regimen was continued for another 20 days; the amount in the blood decreased to 5·1, the urinary output to 9–10 mg. per day. By taking 1750 mg. of ascorbic acid saturation was again reached. The amount in the blood rose to 13·2 mg.

From these experiments it follows that together with the development of a vitamin C deficiency the amounts of ascorbic acid in the blood and in the urine decrease. This agrees with the findings of Abt *et al.* [1936] and Taylor *et al.* [1936] on the ascorbic acid content of blood serum. It appears to be possible to get an insight in the state of saturation of a given subject by determining the ascorbic acid content of the blood or of the urine. For this purpose the estimation in the blood is preferable, for it is less complicated because it needs only one examination whilst the excretion in the urine has to be determined during 24 hours and immediately after voiding. Furthermore there are rather large fluctuations in the daily output of the same subject. Moreover, the daily output is very unequal in different subjects with about the same state of saturation, in the nearly saturated state varying from 9 to 27 mg. The amount in the blood decreases more and more quickly than that of the urine. The relation between

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the state of saturation and the content in the blood however is not linear.[1] For this reason three classifications only appear reasonable: about or above 13 mg./l. signifies saturation, below 4 mg. has to be considered as insufficient, between 13 and 4 mg. more or less sufficient.

In illustration of the facts described above some determinations are given of the ascorbic acid content of the blood before and after saturation with ascorbic acid of three patients probably suffering from scurvy.

In two patients the initial ascorbic acid contents were 3·8 and 2·2 mg./l. After saturation with daily oral doses of 300 and 400 mg. to an amount of 3300 and 4000 mg. ascorbic acid the concentrations in the blood increased to 12·8 and 13 mg./l. In the third case the patient was saturated with 400 mg. ascorbic acid daily given *per os* or intravenously. The amount in the blood was estimated just before the daily dose of ascorbic acid was given, except on 25 February (5 hours after administration). The ascorbic acid content of the urine was investigated during 5 hours after administration of the ascorbic acid. The results are given in Fig. 2.

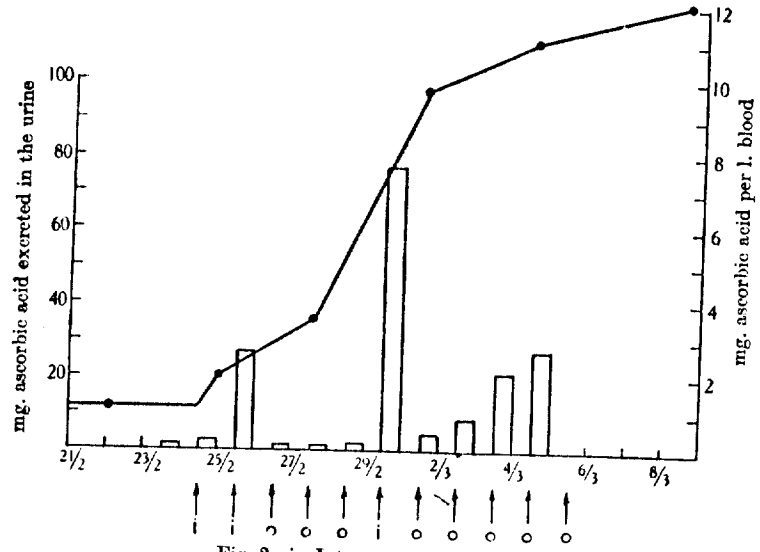

Fig. 2. i = Intravenous. o = per os.

The figure shows that after intravenous administration of ascorbic acid an increased urinary excretion already results before the point of saturation is reached. This can be explained by the supposition that after the injection the amount of ascorbic acid in the blood rises for a short period above the excretion level.

[1] *Note added 4 December 1936.* Determining the concentration of ascorbic acid in the blood and the quantity necessary to saturate different subjects I have found that the relation between these values is linear. A concentration in the blood of 4 mg. per l. corresponds with a saturation dose of about 2 g.; 8 mg. per l. corresponds with a dose of 1 g. ascorbic acid (for subjects with a body weight of 70 kg. The decrease of ascorbic acid in the blood during a dietetic period (see Fig. 1) is not linear because the use of ascorbic acid decreases during this period, as has been demonstrated in this paper.

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The capillary resistance test.

According to Göthlin [1931] and Dalldorf [1933] the capillary resistance depends on the state of saturation; a lowered capillary resistance should indicate a deficiency.

During the first experiment described above some changes in the capillary resistance might have been expected at the end of the dietetic period. A considerable deficiency could be demonstrated by the saturation test (viz. 3250 mg. ascorbic acid had to be taken to reach the point of saturation). Yet the capillary resistance remained normal (see Table III). The technique of the measurements was that given by Göthlin (50 mm. Hg pressure during 15 min.). It appears noteworthy that at the end of the experiment (after 84 days) no other symptoms of vitamin C deficiency than fatigue and irritability could be observed; the body-weight remained constant.

Daily human requirements.

During the first experiment lasting from 6 August to 7 November, 3250 mg. ascorbic acid were taken (Table III). To calculate the amount stored or used in the body from the quantity taken must be subtracted the amount of ascorbic acid excreted above the normal average during the last few days of intake (about 30 mg.). The experiment lasting 94 days the daily requirements amount to

$$\frac{3220}{94} = 34 \text{ mg.}$$

This result differed somewhat from that of a similar informative experiment done in 1934. In this experiment the ascorbic acid content of the urine was estimated directly, without treatment with mercuric acetate and H_2S. This omission did not influence the main result of the experiment because the aim was the registration of a definite peak in the urinary output of ascorbic acid. That experiment lasted 40 days, 1850 mg. were required, the daily requirements amounted to 45 mg.

The difference between these two experiments can possibly be accounted for by their different durations. The decreased urinary output of ascorbic acid at the end of the dietetic period eventually indicates restricted requirements. The requirements should be dependent on the amount of the vitamin stored in the organism. To test this supposition another experiment was done lasting only 27 days, from 7 November to 4 December (Table III, Fig. 1). During this period the organism has not been depleted appreciably (the amount in the blood became 5·1 mg./l.). 1750 mg. minus 38 mg. (surplus excreted) were required to saturate the organism again. From this determination a daily requirement of 63 mg. can be calculated (1712/27). Thus we may suppose an adaptability of the daily requirement to the amount of ascorbic acid stored in the organism. The requirement decreases when the organism becomes depleted. During a period without strongly depleted depots the amount used daily is 63 mg.

According to this method [van Eekelen, 1936] van Wersch [1936, 1, 2] has determined the daily requirements for himself (man 38 years, height 168 cm., weight 72 kg., duration of the experiment 31 days) and for a female patient suffering from degeneration of the skeleton probably caused by chronic scurvy (age 18 years, height 136 cm., weight 53 kg., duration of the experiment 17 days). Requirements amounting to 56 and 44 mg. respectively resulted (the concentrations in the blood after saturation were respectively 13 and 13·7 mg./l.). Evaluating the daily requirements of these three experimental persons per kg. body weight, a close agreement can be observed when the weights are corrected to the body lengths (calculated from Shall's [1933] height-weight-age table). (See Table IV).

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Table IV.

	Height cm.	Weight kg.	Corrected weight	Daily requirements mg. ascorbic acid	Daily requirements per kg.	Daily requirements per kg. corrected weight
Van Eekelen	178	90	74·6	63	0·70	0·84
Van Wersch	168	72	67	56	0·78	0·84
Patient J	136	53	?	44	0·83	0·83

From this the conclusion may be drawn that corpulent people require per kg. body weight less ascorbic acid than slender ones. Possibly the adipose tissues require little ascorbic acid.

From the data presented it follows that the daily requirements for healthy adults weighing 70 kg. amount to about 60 mg.

For a further discussion of the daily human requirements I will refer to the communication of Heinemann in this number of the *Journal* (p. 2299).

Summary.

The content of ascorbic acid in blood and its urinary output depend on the amount of vitamin C taken in and on the quantity stored in the organism. Saturation of the organism coincides with a certain level in the blood (about 13 mg./l.). This level being surpassed surplus excretion in the urine results. To estimate the rate of saturation the determination of ascorbic acid in the blood has proved to be the most reliable method (besides the saturation test). A method to determine daily human requirements for ascorbic acid is described.

The daily requirements are dependent upon the amount stored in the organism, they are largest when the subject does not become markedly unsaturated.

The daily dose required for adults weighing 70 kg. amounts to about 60 mg. under normal conditions.

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Ascorbic Acid in the Lens of the Naphthalene-fed Rabbit

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If the lens of the rabbit fed naphthalene (1 g/kg body weight/day) does not become severely cataractous, the concentration of ascorbic acid within it tends to increase progressively with time. In vitro there is an increase in the ascorbic acid concentration of a lens incubated in a medium containing ascorbic acid and 1,2-dihydro 1,2-dihydroxy naphthalene (naphthalene diol), which is a product of the metabolism of naphthalene by the rabbit (Booth and Boyland, 1949).

1,2-Naphthaquinone, formed in the eye from naphthalene diol and other metabolites by a combination of enzymic and non-enzymic reactions, readily oxidizes ascorbic acid (van Heyningen and Pirie, 1967). It is suggested that the accumulation of ascorbic acid in the lens of the naphthalene-fed rabbit is due to the penetration of dehydroascorbic acid, formed in this way and re-reduced in the lens to ascorbic acid by glutathione. This idea is strengthened by the finding that dehydroascorbic acid itself readily penetrates the lens in vitro.

1. Introduction

Cataract in the naphthalene-fed rabbit is caused by the presence of naphthalene derivatives in the vitreous and aqueous humours (van Heyningen and Pirie, 1967). One of these metabolites is naphthalene diol, i.e. 1,2-dihydro 1,2-dihydroxynaphthalene [Fig. 1(a)]; this is oxidized by an enzyme, catechol reductase (E.C.1.3.1.5) present in the lens and other tissues of the eye, and $NADP^+$* to give NADPH and 1,2-dihydroxynaphthalene [Fig. 1(b)] [equation (1)]; this compound is readily auto-oxidized at neutral pH to yield 1,2-naphthaquinone [Fig. 1(c)] and H_2O_2 [equation (2)]. Other metabolites of naphthalene also react with enzymes in the eye to give 1,2-dihydroxynaphthalene and thence 1,2-naphthaquinone.

$$\text{Naphthalene diol} + NADP^+ \rightarrow \text{dihydroxynaphthalene} + NADPH + H^+ \quad (1)$$

$$\text{Dihydroxynaphthalene} + O_2 \rightarrow \text{naphthaquinone} + H_2O_2. \quad (2)$$

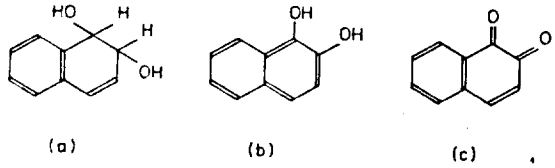

FIG. 1. Formulae of metabolic products of naphthalene metabolism. (a) 1,2-Dihydro,1,2-dihydroxynaphthalene (naphthalene diol); (b) 1,2-dihydroxynaphthalene; (c) 1,2-naphthaquinone.

In the presence of ascorbic acid the following further reaction takes place [equation (3)]:

$$\text{Naphthaquinone} + \text{ascorbate} \rightarrow \text{dihydroxynaphthalene} + \text{dehydroascorbate} \quad (3)$$

* Abbreviations used: GSH, glutathione; GSSG, ozidized glutathione; $NADP^+$ and NADPH, ozidized and reduced coenzyme, nicotinamide adenine dinucleotide phosphate.

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We have shown that these reactions probably take place in the eye of the naphthalene-fed rabbit (van Heyningen and Pirie, 1967), and that the decreased concentration of ascorbic acid in the aqueous and vitreous humours and in the retina is due to its removal by oxidation with naphthaquinone [equation (3)].

We were surprised, therefore, to find that in spite of the loss of ascorbic acid from these tissues, in the non-cataractous lens of the naphthalene-fed rabbit, the concentration of ascorbic acid was increased. This paper describes a study of this phenomenon.

2. Materials and Methods

Treatment of rabbits

Dutch and albino rabbits, usually 3–6 months old, were used. Methods of dosage with naphthalene and examination of the eye have been described previously (van Heyningen and Pirie, 1967).

Most of the lenses used in the experiments described in this paper had no visible changes, or only minimal changes (such as a few water clefts at the periphery or a few subcapsular vacuoles). A few had water clefts all round the periphery.

Lens and aqueous humour were analysed immediately after the rabbit was killed, or they were stored at −20° until required. Weighed lenses were ground with 3 ml 10% (w/v) trichloroacetic acid; after chilling for 1 hr, protein was removed from the extract by centrifugation. To the aqueous humour an equal volume of the trichloroacetic acid solution was added and the precipitate, if any, was removed by centrifugation.

Incubation of rabbit lens

The medium was Krebs Ringer Phosphate, pH 7·0 (Cohen, 1949), containing glucose (0·1% w/v; i.e. 5·6 mM), and ascorbate (20 μl of 0·5 M/10 ml medium; i.e. 1·0 mM). Incubations were in air, at 34°, in lens incubation tubes (Merriam and Kinsey, 1950; van Heyningen, 1965). Ten ml. of medium, containing 3·0 mM naphthalene diol or 0·05–0·1 mM naphthaquinone when appropriate, were put in the incubation tubes in the water-bath. The rabbit was killed and each lens slipped into its prepared tube. Samples of each incubation medium (50 μl) were withdrawn at intervals of 15 or 30 min and assayed for ascorbic acid. If the concentration had fallen appreciably a calculated volume of 0·5 M ascorbate was added to bring the level back to approximately 1·0 mM. At the end of the experiment the lenses were removed, rinsed briefly with distilled water and added to a weighed tube containing 3·0 ml 10% (w/v) trichloroacetic acid. The tube was re-weighed and the weight of the lens was obtained by difference. The lenses were disintegrated with a glass rod and the mixture chilled for about 1 hr; the protein was then removed by centrifugation and the extract assayed for ascorbic acid and GSH.

Dehydroascorbic acid (20 μl of 0·5 M), when used, was added to the incubation medium just after the lens, giving a final concentration of 1·0 mM. On some occasions the whole medium was changed one or more times at hourly intervals. In this case the medium in the tube was carefully poured out, and 10 ml fresh medium, at 34°, to which the dehydroascorbic acid had just been added, was substituted. The reason for this manoeuvre is the lability of dehydroascorbic acid at pH 7·0. Its half-life at pH 7·24 and 30° is about 5 min (Ball, 1937; see also Langham and Heald, 1956).

Estimations

Ascorbic acid was determined by titration with 2,6-dichlorophenol indophenol and total ascorbic acid by the method of Roe (1954). Dehydroascorbic acid was taken to be the difference between these two measurements. Thiol groups were determined by the method of Ellman (1959). Total thiol was assayed in a lens extract, GSH in the supernatant after removal of protein with trichloroacetic acid, and protein thiol was taken as the difference

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between these two values. GSSG was assayed spectrophotometrically, using glutathione reductase (Bergmeyer, 1963).

Materials

1,2-Dihydro 1,2-dihydroxynaphthalene was a gift from Professor Sir Ewart Jones and 1,2-naphthaquinone from Mrs J. Rees. Dehydroascorbic acid (0·5 M) was made by oxidizing ascorbic acid with bromine, as follows. Ascorbic acid (176 mg) was dissolved in 2 ml water in an ice-bath, in a fume cupboard. Bromine was added (about 0·06 ml) until a faint yellow colour persisted: this was removed by adding a speck of ascorbic acid and N_2 was then bubbled through the solution for several minutes to remove all traces of bromine. It was freshly prepared for each experiment. Ascorbic acid, 0·5 M, neutralized to about pH 6·8 with NaOH and kept in ice, was freshly prepared for each experiment.

Reaction between dehydroascorbic acid and thiols

Bovine lens was used for this experiment. Four lenses (8 g) were ground with 16 ml of 0·1 M phosphate buffer, pH 7·4. Insoluble protein was removed by centrifugation and the extract was then dialysed for 3 days at 4° against distilled water. The water was stirred continuously and changed twice daily. The contents of the dialysis sac were frozen for 2 days, then thawed and diluted with an equal volume of 0·1 M phosphate buffer pH 7·0. To 10 ml of this 1 in 6 lens extract was added 4·4 mg of GSH to give a concentration of 1·46 mM. Samples were withdrawn from the lens extracts, with or without added GSH, for assay of initial protein and GSH thiol; to 5 ml of each, at 37°, was added 0·04 ml of 0·5 M DHA and samples were withdrawn at intervals over the next hour for assay of thiol and ascorbic acid.

3. Results

In vivo experiments

Ascorbic acid in aqueous humour and lens. Table I compares the concentration of

TABLE I

Effect of naphthalene feeding on the concentration of ascorbic acid in the aqueous humour of the rabbit

Rabbit no.	No. of doses of naphthalene	Ascorbic acid in aqueous humour (μmoles/ml) Naphthalene-fed	Control	% difference
43	2	0·17	1·02	−83
1	2	0·16	1·13	−90
12*	3	1·02	1·96	−48
32	3	0·25	1·10	−77
2	5	0·45	1·59	−72
33	9	0·31	0·77	−60
29*	9	0·48	1·07	−55
35*	10	1·36	1·90	−28
36	10	0·99	1·90	−48
30*	15	1·47	2·18	−33
16†	23	0·23	0·94	−76
14	28	1·99	1·14	−75

Naphthalene (1 g/kg body wt) was administered daily.
Each comparison is between littermates.
* Water clefts around periphery of lens; in the remainder the lenses showed no, or minimal change.
† Protein in aqueous humour, severe cataract.

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ascorbic acid in the aqueous humour of the naphthalene-fed rabbit with that of an untreated littermate. The normal values range from 0·77 to 2·18 μmoles/ml (average 1·35 μmoles/ml). With one exception the concentration in the aqueous humour of the naphthalene-fed rabbit was lower than that in the untreated rabbit. However, after the second dose the difference between the levels in the treated and untreated animals

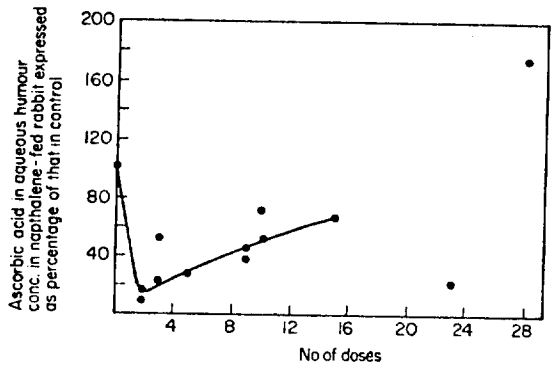

FIG. 2. Ascorbic acid in the aqueous humour of the naphthalene-fed rabbit. The percentage difference between the concentration of ascorbic acid in the aqueous humour of the naphthalene-fed rabbit, and that in a littermate control, is plotted against the number of daily doses of naphthalene.

tended to decrease as the number of doses increased (Fig. 2); the concentration of ascorbic acid in the aqueous humour of a rabbit which had had 28 doses was higher than that in the control. The one value which does not fit into this pattern, −76% in rabbit no. 16 after 23 doses, was obtained from an aqueous humour which in vivo showed a strong flare. Secretion of ascorbic acid by the ciliary body may therefore have been impaired.

TABLE II

Ascorbic acid in the serum of a naphthalene-fed rabbit

No. of doses	Ascorbic acid (μmoles/ml)
0	0·053
3	0·069
6	0·102
9	0·108
13	0·120

Rabbit no. 45—naphthalene (1 g/kg) was administered daily.

Since there is some evidence that naphthalene-feeding brings about an increase in the concentration of conjugated glucuronic acids in the blood and in the aqueous and vitreous humours (Takagi and Okumura, 1961; Kiuchi, 1959), the gradual return to

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normal values with increasing numbers of doses of naphthalene (Table I and Fig. 2) may be because less unconjugated metabolic products of naphthalene reach the eye. Detoxication by increased synthesis of glucuronides is often accompanied by increased synthesis of ascorbic acid and, in a single experiment, this was shown to occur in naphthalene feeding. Blood, withdrawn from a naphthalene-fed rabbit at intervals, was assayed for total ascorbic acid by the method of Roe (1954). Table II shows that, over a period of 13 days, the concentration increased rather more than twofold. The amount of ascorbic acid secreted into the aqueous humour would therefore also be increased (Kinsey, 1947). Formation of glucuronides may therefore act in two ways, to bring about a gradual increase in the ascorbic acid of aqueous humour of the rabbit, dosed daily with naphthalene: (1) by partial removal of toxic naphthalene metabolites; and (2) by increased synthesis of ascorbic acid.

TABLE III

Effect of naphthalene-feeding on the concentration of ascorbic acid in the lens of the rabbit

Rabbit no.	No. of doses of naphthalene	Ascorbic acid in lens (μmoles/g) Naphthalene -fed	Control	% difference
1	2	1·14	1·14	0
12*	3	2·74	1·27	+116
32	3	1·36	1·19	+14
2	5	1·76	1·25	+41
31*	5	3·22	1·15	+180
13*	8	2·40	1·26	+91
39*	8	2·97	1·54	+93
29*	9	2·42	1·68	+44
21	9	2·92	1·61	+81
33	9	1·66	1·16	+43
35*	10	3·70	1·19	+211
36	10	3·01	1·19	+150
30*	15	1·29	1·39	−9
14	28	4·55	1·76	+158

Naphthalene (1 g/kg body wt) was administered daily.
Each comparison is between littermates.
* Water clefts around periphery of lens; the other lenses showed no, or minimal changes.

Table III compares the concentration of ascorbic acid in the lens of the naphthalene-fed rabbit with that of its untreated litter mate. Whereas there is a deficit of ascorbic acid in the aqueous humour of the naphthalene-fed rabbit (Table I and Fig. 2) in the lens ascorbic acid accumulates. The average value for the ascorbic acid in the 14 normal lenses is 1·37 μmoles/g (range 1·14–1·76). In the 14 lenses of the naphthalene-fed rabbits the average value is 2·37 (range 1·14–4·55). There was a tendency for the concentration in the lens of the treated rabbit, compared with the untreated, to increase with the number of doses. (The lens of rabbit no. 30, fed 15 days, is a marked exception.)

A clear-cut difference between the eye of the naphthalene-fed and the control rabbit is shown in Fig. 3. In the normal eye the concentration of ascorbic acid in the lens varies from 1·14 to 1·76 (av. 1·335), while the concentration in the aqueous humour

ranges from 0·77 to 2·18 (av. 1·32 μmoles/ml). In the lens of the naphthalene-fed rabbit concentrations of ascorbic acid which fall within the normal range are associated with low levels in the aqueous humours (Fig. 3). Values for ascorbic acid in the aqueous humour approaching control values are associated with elevated lens concentrations. In the normal lens the ratio $\frac{\text{lens ascorbic}}{\text{aqueous ascorbic}}$ was $1{\cdot}01 \pm 0{\cdot}12$; in the naphthalene-fed rabbit the ratio was $4{\cdot}22 \pm 0{\cdot}75$. In calculating the ratio for the naphthalene-fed rabbit, one value which falls far from the others and among those from the eyes of normal rabbits has been omitted. It corresponds to rabbit no. 30 in which the value for lens ascorbic acid was exceptionally low (Table III).

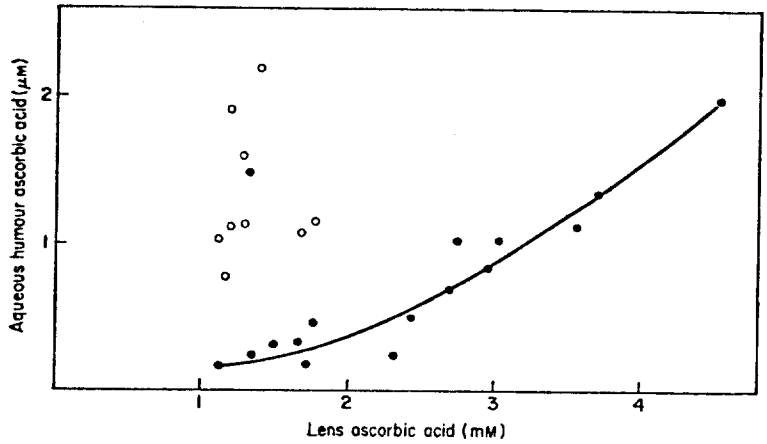

FIG. 3. Relationship between the concentration of ascorbic acid in the lens and in the aqueous humour of the eyes of naphthalene-fed and untreated rabbits. ○, Untreated rabbits; ●, naphthalene-fed rabbits. Values for all eyes in which ascorbic acid was assayed in both lens and aqueous humour are plotted in this figure. The number of doses of naphthalene ranged from 2 to 28.

Thus in the eye of the naphthalene-fed rabbit the concentration of ascorbic acid in the lens was more than 4 times that in the aqueous humour, whereas in the normal eye the average values for lens and aqueous humour were almost the same.

Administration of naphthalene diol in the form of eye drops. Naphthalene diol was administered in the form of eye drops to the right eye of four rabbits (van Heyningen and Pirie, 1967). In three cases there were cataractous changes, but the lenses were not analysed. In the fourth rabbit there were no lens changes, but in the right eye the lens was found to contain more ascorbic acid, and the aqueous humour less ascorbic acid, than the untreated left eye. The values were, for the right and left lens, 2·1 and 1·5 mM and for the right and left aqueous humours 1·3 and 1·65 mM.

Generally, only reduced ascorbic acid in the lens was measured. On a number of occasions, however, total ascorbic acid was assayed (reduced plus dehydroascorbic acid) and it was shown that little dehydroascorbic acid was present in the lens. This is not surprising, in view of the high concentration of thiol groups, of glutathione and protein in the lens.

Glutathione. It is well known that dehydroascorbic acid is rapidly reduced by glutathione, with the formation of ascorbic acid and oxidized glutathione [equation (4)]. This reaction is non-enzymic. There is no evidence that the enzyme which catalyses the reaction in some animal tissues (Hughes, 1964) is present in the lens (Hughes,

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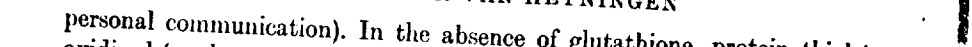
personal communication). In the absence of glutathione, protein thiol is similarly oxidized (see later, Fig. 4):



dehydroascorbate + 2 GSH → ascorbate + GSSG.

Table IV shows that, in the non-cataractous lens of the naphthalene-fed rabbit, the concentration of glutathione was not greatly diminished. With one exception (rabbit no. 13) the concentration of GSH was decreased by 20% or less, compared with the control.

TABLE IV

Effect of naphthalene-feeding on the concentration of glutathione in the lens of the rabbit

Rabbit no.	No. of doses of naphthalene	Glutathione in lens (μmoles/g) Naphthalene-fed	Control	% difference
1	2	11·6	13·1	−11
12*	3	9·0	11·3	−20
32	3	11·0	10·5	+5
2	5	9·3	10·2	−9
31*	5	10·5	11·2	−6
13*	8	6·5	11·2	−42
39*	8	9·5	11·7	−19
29*	9	9·4	11·6	−19
21	9	10·1	9·8	+3
33	9	9·8	12·0	−18
35*	10	10·9	11·0	−1
36	10	11·5	11·0	+5
30*	15	8·5	9·5	−10
14	28	7·8	8·9	−12

Naphthalene (1 g/kg body wt) was administered daily.
Each comparison is between littermates.
* Water clefts around periphery of lens; the other lenses showed no, or minimal changes.

In vitro experiments

Incubation of the lens with naphthalene derivatives and ascorbic acid. To investigate further the increase in concentration of ascorbic acid in the lens of the naphthalene-fed rabbit, the phenomenon was reproduced as far as possible in vitro. The two lenses of a rabbit were each incubated in a medium in which the concentration of ascorbic acid was maintained at the level of about 1 mM (see Materials and Methods); addition of a naphthalene derivative was made to the medium in which one of the lenses was incubated. After some hours the experiment was stopped and the two lenses assayed for ascorbic acid and glutathione. Table V, line A gives the results of these experiments. When the lens was incubated with 3 mM naphthalene diol the ascorbic acid in the medium was oxidized. The medium became brown in colour and the substance of the lens (but not the capsule) also became coloured. Water clefts appeared at the equator, similar to those seen in the lens of the naphthalene-fed rabbit in vivo. The ascorbic acid content of the lens incubated in diol increased in all four experiments by an average of 21%, whereas that of glutathione decreased to 50% (Table V, line A). In one of the four experiments GSSG and dehydroascorbic acid were assayed and both were found to be absent.

It seemed probable that the effect of naphthalene diol, in bringing about the accumulation of ascorbic acid in the lens, was due to the formation and penetration of dehydroascorbic acid [equations (1)-(3)]. To test this, lenses were incubated in a medium containing dehydroascorbic acid, and compared with lenses incubated in ascorbic acid, and also with unincubated lenses.

Table V, line B, shows that, with dehydroascorbic acid in the medium, the concentration of ascorbic acid in the lens increased from 1·13 to 1·96 mM, and there was no change in the level of GSH. Table V, line C, gives the average level of ascorbic acid and GSH in five lenses incubated in millimolar ascorbic acid. These values are close to those of the unincubated lens (Table V, line D).

TABLE V

Effect of naphthalene metabolites and ascorbic acid in the incubating medium on the concentration of ascorbic acid and glutathione in the lens

	Av. age of rabbit (years)	Supplement	Concn (mM)	Length of incubn (hr)	No. of expts	Final concentration in lens (average values) Ascorbic acid (μmoles/g) Expt	Control	% Diff.	Glutathione (μmoles/g) Expt	Control	% Diff.
A	>1	Naphthalene diol	3·0	5	4	1·08	0·89	+21	4·8	9·6	−50
B	0·5	Dehydroascorbic acid	1·0	2–4·5	3	1·96	1·13	+74	11·1	11·2	−1
C	0·5	Ascorbic acid	1·0	4	5	1·17			11·0		
D	0·5	Unincubated	—	—	14	1·37			10·9		
E	>1	Naphthaquinone	0·05–0·10	2·5	3	0·59	0·75	−21	5·4	9·3	−42
F	>1	Naphthaquinone +catalase*	0·09	2·5	2	1·43	0·93	+54	6·6	10·0	−34
G	>1	Dihydroxy naphthalene+ catalase*	0·09	2·5	2	1·20	0·80	+50	8·5	11·7	−27
H	>1	Catalase†	—	2·5	2	0·90	0·88	+2	12·7	12·0	+6

Lines A, E, F, G and H: pairs of rabbit lenses were incubated at 34° in medium containing ascorbic acid and glucose, one with and one without supplements, as shown: The following extra amounts of ascorbate were added to the medium containing supplement to keep the concentration at about 1 mM:
line A, 200 μl; line E, 180 μl; line F, 200 μl; line G, 180 μl; lines C and H, none; to the control media less than 30 μl was added in all cases; line B, ascorbic acid omitted from medium; control lens unincubated.
* 1 mg in expt and control media.
† 1 mg in expt medium only.

It was at first not possible to show that the presence of naphthaquinone in the medium would bring about accumulation of ascorbic acid in the lens; ascorbic acid in the medium was rapidly oxidized but there was a decrease in the concentration of both ascorbic acid and GSH in the lens (Table V, line E).

It seemed possible that a substantial amount of dehydroascorbic acid was penetrating the lens without being reduced to ascorbic acid, but this was shown not to be so. The concentration of dehydroascorbic acid in these lenses was found to be about 0·09 μmoles/g, only about 15% that of ascorbic acid. However, when the enzyme catalase was added to the medium to remove H_2O_2 formed [equation (2)], both naphthaquinone and dihydroxynaphthalene were found to increase the concentration of ascorbic acid in the lens (Table V, lines F and G). The concentration of glutathione was diminished. Catalase alone (Table V, line H) had no effect. The influence of

catalase on the concentration of ascorbic acid in the lens incubated in naphthalene diol was not studied.

H_2O_2 readily enters the lens (Pirie, 1965) and owing to the presence of glutathione peroxidase (E.C.5.3.1.1) (Pirie, 1965; Holmberg, 1968), oxidizes GSH to GSSG [equation (5)]:

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O. \qquad (5)$$

The loss of GSH from the lens incubated in naphthaquinone was not greatly diminished when catalase was added and it is therefore not clear how H_2O_2 prevents the penetration of dehydroascorbic acid into the lens.

Reaction between dehydroascorbic acid and lens thiols. The concentration of glutathione is decreased in the lens of the naphthalene-fed rabbit (Table IV) and in the lens incubated in a medium containing ascorbic acid with naphthalene metabolites (Table V). This is presumably due to the oxidation of thiol group by dehydroascorbic acid [equation (5)], naphthaquinone, and H_2O_2 (in the absence of catalase).

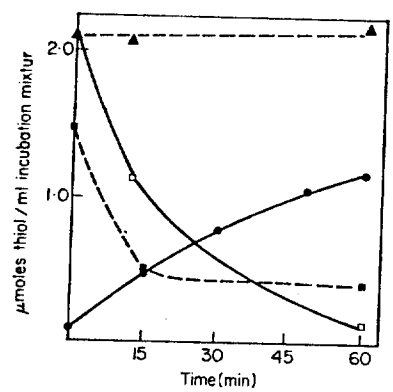


FIG. 4. Reaction between dehydroascorbic acid and protein thiol in the presence and absence of glutathione.

A dialysed lens extract was incubated at pH 7·0 and 37° with dehydroascorbic acid (4 μmoles/ml) with and without added GSH. Samples were withdrawn at intervals and assayed for thiol and ascorbic acid.

■ - - - - ■, Glutathione; ▲ - - - - - ▲, protein thiol; incubation in the presence of GSH; □———□, protein thiol; ●———●, ascorbic acid; incubation in the absence of GSH.

To find out if dehydroascorbic acid also oxidizes protein thiol, a dialysed lens extract was divided into two parts, to one of which was added GSH, at approximately its concentration before dialysis. Both parts were incubated with dehydroascorbic acid. Figure 4 shows that dehydroascorbic acid reacts preferentially with GSH. In the presence of GSH, protein thiol is not oxidized, but in its absence protein thiol is quite rapidly oxidized, and the ascorbic acid formed after 1 hr (1·06 μmoles) is about half the amount of thiol removed (2·06 μmoles) as expected from equation (5). Presumably in these nearly normal lenses of naphthalene-fed rabbits (Table III) the presence of GSH protects protein thiol from oxidation. In the severely cataractous brown lens (van Heyningen and Pirie, 1967; Rees and Pirie, 1967), however, the thiol group of protein may be oxidized by dehydroascorbic acid, as well as combining with naphthaquinone.

The conclusion that the protein thiol of the lens appears to reduce dehydroascorbic acid has been reached by others (Gloster, 1956; Langham and Heald, 1956), but until recently (Ellman, 1959) there has not been a convenient, reliable method of assaying protein thiol.

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4. Discussion

There are a few reports in the literature of the measurement of the ascorbic acid content of the lens of the naphthalene-fed rabbit. Duke-Elder (1954) summarizes "... it is interesting that a loss of vitamin C is a late phenomenon in naphthalene cataract". Nordmann (1954) says that vitamin C decreases in the aqueous humour a few hours after naphthalene feeding, but is unchanged in the lens; it decreases slowly, from the first sign of cataract, and finally the values are very low. I have found no report of an increase in lens ascorbic acid as a result of naphthalene feeding.

It should be stressed, however, that none of the lenses of the naphthalene-fed rabbits examined in this paper were seriously cataractous. Some had water clefts around most of the periphery, some had a few water clefts, subcapsular vacuoles, or other slight changes, and the rest appeared quite normal. Ascorbic acid is not at a high concentration in the severely cataractous and therefore probably "leaky" lens. In the aqueous humour of rabbit no. 16 (Table I), in which the cataract was advanced, ascorbic acid was at a much lower concentration than others in the series; this low level would be associated with a low concentration of lens ascorbic acid (Fig. 3), even in a fairly normal lens.

Dehydroascorbic acid is formed in the eye [equation (3)] (van Heyningen and Pirie, 1967), readily penetrates the lens in vitro, and there becomes reduced to ascorbic acid (Table V, line B). Ascorbic acid accumulates in the lens incubated in a medium containing diol and ascorbic acid (Table V, line A). It seems likely, therefore, that dehydroascorbic acid formed in the aqueous humour of the naphthalene-fed rabbit [equation (3)] penetrates the lens and is there reduced to ascorbic acid [equation (5)], which does not easily escape. It has been shown that ascorbic acid diffuses fairly slowly out of the lens (Wachtl and Kinsey, 1958), so that the continuous formation of a low concentration of dehydroascorbic acid in the aqueous humour could account for the rise in concentration of ascorbic acid in the lens, as a result of naphthalene feeding.

Dehydroascorbic acid, being more lipid-soluble than ascorbic acid, is known to penetrate many cells more readily than ascorbic acid (Patterson and Mastin, 1951; Räihä, 1956; Martin, 1961). The question of whether the entry of dehydroascorbic acid into the lens is an "active" process is left open, since passive diffusion from a region of higher (the aqueous) to lower (the lens) concentration would account for the phenomenon.

This is the only report of an increase in concentration of ascorbic acid in the lens as a result of administration to the animal of a foreign substance. So far it has only been brought about in vivo by naphthalene feeding or by applying naphthalene diol directly to the eye in the form of eye drops. Decrease in ascorbic acid has been reported to occur in many types of cataract, though not usually in a regular or consistent manner.

Glutathione. In the lens of the naphthalene-fed rabbit the concentration of GSH was, with one exception, decreased by no more than 20% and often by much less (Table IV). GSSG is formed in the lens when GSH is oxidized with dehydroascorbic acid and naphthaquinone. H_2O_2, formed when 1,2-dihydroxynaphthalene is autoxidized [equation (2)] oxidizes GSH enzymically [equation (5)]. The concentration of glutathione in the non-cataractous lens is kept high in spite of all these oxidative reactions.

The explanation is presumably the presence of the enzyme glutathione reductase

[equation (6)], which, in the normal lens, is probably responsible for a large part of the reoxidation of NADPH formed in the pentose phosphate pathway:

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+. \quad (6)$$

In the lens of the naphthalene-fed rabbit, GSSG and naphthaquinone presumably compete for this coenzyme.

In the explanted lens there is a considerable loss of GSH. Naphthaquinone (Table V, lines A,E,F,G) and H_2O_2 (Table V, lines A and F) are both at far higher concentrations than in vivo, and penetrating the lens together with dehydroascorbic acid will rapidly oxidize GSH to GSSG. Naphthaquinone will also oxidize NADPH and thus inhibit the effective reoxidation of GSSG by glutathione reductase [equation (6)]. In the absence of the quinone, and of H_2O_2, dehydroascorbic acid penetrates the lens and is reduced to ascorbic acid with no resultant loss of GSH (Table V, line B).

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A BIOCHEMICAL ABNORMALITY IN SCHIZOPHRENIA INVOLVING ASCORBIC ACID

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ABSTRACT

Clinicians have reported that ascorbic acid in large dosage has therapeutic value in the treatment of schizophrenia. This observation is consistent with the known physiological functions of this vitamin. A clinical laboratory test was devised to indicate the ratio of utilization of ascorbic acid in normals and schizophrenics. It was found that schizophrenics could metabolize ten times as much of this vitamin as normals. The therapeutic response was selective, which could have been predicted since only the capacity to function is altered. A mature behavior pattern depends on experience and learning, which obviously this vitamin cannot furnish.

Several clinicians have observed that ascorbic acid in large dosage has a therapeutic effect in patients with schizophrenic reaction.[1] This suggests a biochemical abnormality in the utilization of this vitamin in these patients. This observation is consistent with the known biochemical action of this vitamin. A deficiency causes a marked lowering of many essential enzymatic reactions. Specific metabolic abnormalities associated with a deficiency of ascorbic acid, particularly in the metabolism of tyrosine, can readily be demonstrated.[2] The abnormal metabolites thus produced have been found in the urine of schizophrenics.

Ascorbic acid occurs in high concentrations in the medulla of the adrenal gland. It is essential in the formation of noradrenalin and adrenalin. These hormones are active and essential in metabolic processes of the nervous system.[3] Since humans cannot synthesize this vitamin and can store it in only limited amounts, a defect in the utilization of it could cause serious malfunctioning of the body economy.

Tests for the detection of ascorbic acid in the blood plasma and urine are readily available; however, they can be very misleading. The concentration of ascorbic acid in the adrenal medulla is much higher than in blood plasma or urine. Utilization of this vitamin and the tissue concentration cannot be expressed accurately in terms of mg. per cent of blood serum. The situation is very similar to that of evaluating utilization of antibiotics. Here also blood levels do not correlate well with clinical effectiveness. A laboratory method must be devised which would indicate tissue utilization rather than serum concentration. Such a test can be devised since an intake of excessive amounts of ascorbic acid has no deleterious effects in man. The ascorbic acid that is not metabolized is excreted as such in the urine.

Laboratory Procedure:

If one tenth milliliter of commercial thorazine concentrate is added to one milliliter of Forrest Reagent, the solution acquires a deep cherry red color.[4] This solution contains 20 parts of 5% ferric chloride and 80 parts of 10% sulphuric acid. If then one tenth milliliter of a saturated solution of ascorbic

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acid is added, the solution becomes colorless instantly. This reaction can thus be used to demonstrate the utilization of ascorbic acid. When an excessive amount of ascorbic acid is ingested, the unmetabolized portion appears in the urine. If one milliliter of urine were added to the above cherry red solution and the above noted color change occurred instantly, the presence of unmetabolized ascorbic acid would be indicated. This amount of urine was selected for purposes of standardization of the laboratory procedure. If it were determined that schizophrenics required a much larger amount of ascorbic acid than normals to effect this color reaction, a difference in the utilization of this vitamin would be demonstrated. It would be in terms of a ratio rather than of an absolute value expressed in grams of ascorbic acid excreted.

METHOD

This test was given to eight normal male employees and ten chronic schizophrenic patients. Ascorbic acid was given in divided doses six times a day at four hour intervals. The urine was examined for ascorbic acid by the above described method immediately after voiding. The amount of ascorbic acid ingested was increased daily until one milliliter of urine would cause the cherry red color of the test solution to disappear instantly. This reaction was obtained in normals when they received from one-half to one gram of ascorbic acid every four hours. The average total dose for the eight normal employees was four grams daily. However, to obtain the same reaction with the test solution, the patients with schizophrenic reaction required from six to eight grams per dose every four hours or a total of 36 to 48 grams a day. The average for the 10 patients was 40 grams. Thus the ratio between the normals and schizophrenics was as four is to 40. In other words, patients with schizophrenic reaction can metabolize ten times as much ascorbic acid as normals. The normals used in this research project were employees at this hospital. The patients were male chronic schizophrenics. All of them had been hospitalized more than two years, several more than ten years. Ascorbic Acid in dosage of 36 to 48 grams daily for therapeutic effect was also given to a representative group of similar patients by various physicians of the staff of this hospital. The social adjustments these patients had made prior to hospitalization ranged from living on skid row to college graduation.

RESULTS

In assessing a deficiency of ascorbic acid, the most reliable test still is the clinical observation of prompt improvement following the administration of this vitamin in adequate doses.[5] All the patients who received this vitamin in these large doses made a definite clinical improvement. This was shown chiefly in the area of socialization. The patients expressed a feeling of well being. The anxious, tense facial expression was replaced with a smile and friendliness. They stated that they didn't feel so "hemmed in," "People didn't seem to be so against me," "I can now think more clearly". Those who were shy, seclusive and withdrawn began to participate in ward activities, in conversation with other patients and ward personnel. The improvement however was still marginal.

EPICRISIS

Therapists most likely will be disappointed in the results obtained with ascorbic acid. On closer analysis the improvement is what could have been expected. The vitamin can only alleviate the chemical and metabolic abnormality. It can improve the capacity to function. It cannot furnish learning and experience, the essential ingredients in ego maturation and integration.[6] No doubt many chronic schizophrenics have experienced such prolonged deprivation of these necessary elements that they will require a very structured environment. Continued hospitalization for an indefinite period of time will be necessary. Ascorbic acid can make the experience and learning in this structured environment more pleasant and more meaningful.

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Unusual Complication of Excessive Ingestion of Vitamin C Tablets

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A 66-year-old white woman presented with complaints of a "dead aching pain, cramplike" in the right lower quadrant of her abdomen, associated with a sensation of nausea. This pain was intermittent and not aggravated by the intake of food. There had been no chills, fever or change in bowel habits.

Her family history revealed that her father died of uremic poisoning, one sister died at age 10 with uremic poisoning and another sister died at age 29 of Bright's disease. The patient herself has had one kidney removed due to a large staghorn calculus which occluded the kidney and rendered it non-functioning.

Physical Examination. — The well-developed, well-nourished, white female appeared to be several years younger than her stated age of 66 years. Vital signs on admission were all within normal ranges. No abnormalities were noted of the head, neck, heart or lungs. Examination of the abdomen revealed an ill-defined mass in the right lower quadrant with associated tenderness but no muscle guarding. There was reduction of the normal peristaltic activity with on-rushes of peristaltic activity appearing slightly hyperactive at times. Bimanual pelvic examination revealed an unusual amount of tenderness with a vague mass in the right adnexa and cul-de-sac area. This mass appeared to be nodular and tender.

Radiological and Laboratory Data. — Complete blood cell count was within normal limits with 5,600 white blood cells and 43% hematocrit. Urinalysis was unremarkable. Chest x-ray showed degenerative changes; otherwise it was normal for her age. Barium enema x-ray results were entirely negative. The kidney-ureter-bladder intravenous pyelogram revealed some slight opaque shadows consistent with medication. The intravenous pyelogram itself was normal with surgical absence of the left kidney.

Surgical and Pathological Findings. — Because of the physical findings and the patient's persistent complaints of pain, exploratory laparotomy was undertaken with a preoperative diagnosis of ovarian tumor or tubo-ovarian abscess. The abdomen was opened through a standard midline incision. The small intestine from the ileocecal valve to a point just below the jejunum contained numerous hard stony objects ranging in circumference from large marbles to smaller than aspirin. Because we could not identify these objects, an incision was made into the lower ileum and a dozen or so of the larger ones were removed. These stones were located immediately adjacent to the valve and appeared to be obstructing it. None of the material could be found in the cecum. Grossly, this substance appeared to be some type of medication. The area around the valve and as much of the distal segment of the small bowel as could be reached were carefully cleared of all this debris. In the process, approximately 50 such stony objects, varying in size, were removed. Similar material remained scattered throughout the ileum, however, it had a soft flaky consistency and we felt this would pass successfully, so no attempt was made to remove it. The ileum was then closed transversely with a 00 atarumatic catgut running lock suture, then reinforced with Lembert 4-0 black silk seromuscular type suture. An incidental appendectomy was performed and the abdomen was then closed in a routine manner.

Chemical analysis of the material removed from the ileum revealed amorphous material containing 106 mg/100 ml of ascorbic acid (normal values are 0.2 to 2.0 mg/100 ml).

Discussion

The patient and her husband were advised of the findings at surgery. At this point they revealed a long history of ingestion of various types of vitamins. Their average *daily* intake of vitamin preparations for the past approximately nine years was as follows: 100-200 mg of vitamin E, 50,000 units of vitamin A, four tablets of vitamin B (brewer's yeast), 30 gr of bone meal and 300 mg of vitamin C. Occasionally massive doses of vitamin C were ingested consisting of five 300 mg tablets three times a day. The patient revealed that she had had mild symptoms of a cold approximately two weeks prior to her present illness and had taken 15 vitamin C tablets for four or five days; thereafter she resumed her customary tablet per day. When she began to experience the abdominal pain and discomfort described in the present illness, she repeated this regime of 15 tablets per day, but when she did not improve she sought medical attention. Further, she stated that when she learned her problem would necessitate surgery she took 30 additional vitamin C tablets during the 24 hours prior to her surgery.

This program of vitamin intake was carefully discussed with the patient and her husband, with a special caution concerning the excessive intake of vitamin C on the patient's

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part, in view of her history of renal calculi. She was discharged home on the seventh postoperative day in satisfactory condition.

The vitamin C controversy has been widely discussed. Pauling[1] states that: "The regular use of ascorbic acid in the optimum daily amount appropriate to you as an individual human being may suffice to keep you from catching the common cold or influenza or other infection, under most circumstances." Dr. Pauling recommends 1-2 gm (1000-2000 mg) of vitamin C daily for the average person (with up to 5 gm for some individuals). If symptoms of a cold should develop despite this therapy, Dr. Pauling recommends up to 10-15 gm per day. This amount contrasts sharply with the National Research Council recommendation of approximately 70 mg as the maximum daily adult dose.

According to Cooper[2], when tissues reach saturation level, the excess ascorbic acid is excreted in the urine. It is generally believed that a habitual intake of vitamin C of between 80 and 100 mg daily will keep a person in a state approaching saturation. If this is the case, it would appear that extra intake of vitamin C would be rendered useless by the body systems themselves.

Another report[3] warns of possible precipitation of urate and cystine stones in the urinary tract with high dosages of vitamin C, as well as stating: "Nor can the possibility of other adverse effects of very large doses be excluded."

Summary

A case is presented of ingestion of massive dosages of vitamin C tablets causing an intestinal obstruction at the ileocecal valve. At surgery, multiple hard, rock-like objects varying in size from large marbles to aspirin were found in the ileum, with the larger ones occluding the ileocecal valve. Subsequent chemical analysis of this material revealed it to be primarily vitamin C.

Resumen

Se presenta el caso de ingestión masiva de tabletas de Vitamina C que produjeron obstrucción intestinal a nivel de la válvula ileocecal. En la operación se encontraron múltiples objetos duros con aspecto de rocas, variando en tamaño desde el de marbles grandes hasta el de una tableta de aspirina, a nivel del ileum; las más grandes ocluían la válvula ileocecal. El análisis químico de este material demostró que se trataba esencialmente de Vitamina C. (A.V.)

Zusammenfassung

Ein Fall von Ingestion massiver Dosen von Vitamin C Tabletten mit intestinaler Obstruktion der ileozäkalen Klappe wird präsentiert. Bei der Operation wurden viele harte, steinförmige Objekte, in Grösse von grössen Kugeln bis zu Aspirin Tabletten im Ileum gefunden. Die grösseren verschlossen die ileozakäle Klappe. Eine chemische Analyse des Materials ergab, dass es meistens Vitamin C was. (E.J.O.)

Riassunto

Si presenta un caso di ostruzione intestinale a livello della valvola ileocecale per ingestione massiva di pastiglie di Vitamina C. All'operazione furono trovate molte concrezioni dure come sassi di grandezza varia, da grandi palline a piccole aspirine colle più grosse ostruenti la valvola ileocecale. Esami chimici di queste concrezioni rivelarono che erano formate pricipalmente di Vitamina C. (C.M.)

Résumé

On présenté un cas d'ingestion massive de comprimés de Vitamine C, causant une obstruction intestinale de la valve ileo-caecale. A l'opération on trouva dans l'ileum plusieurs objects durs comme du roc dont la grosseur variait entre une comprimé d'aspirine et une grosse bille ceux-ci occluant la valve ileo-caecale. L'analyse chimique subséquente de cette substance révéla qu'il sagissait principalement de vitamine C. (C.M.)

Sumário

Um caso de obstrução intestinal é apresentada após ingestão maçiça de vitamina C comprimidos. A valvula ileocecal, na cirurgia foi encontrada obstruida por formações calcareas, duras e em diferentes tamanhos. O exame quimico revelou tratar-se realmente de Vit. C. (J.O.C.X.)

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Hypervitaminosis C and Hematic Variations
(Changes in Blood Composition)

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Introduction

Vitamin C or ascorbic acid, isolated in 1932 by Szent-Gyorgyi and considered by Naworth and Micheel, in regard to its chemical structure, as the oxidation product of the hexose, l-gulose, and more precisely as the anhydride of beta-ketogulonic acid, was synthesized by Reichskin et al. in 1934. Active only it its levorotary form (l-ascorbic acid), it is a white crystalline powder, easily soluble in water and imparting to it an acid reaction; it is stable in the dry state, but is readily oxidized in solution and converted into dehydroascorbic acid. Such a characteristic property is due to the presence of two enol groups united by a double bond; the above reaction is reversible, and the vitamin thus functions as an oxidation-reduction system, and as such takes part precisely in the most varied functions of the organism.

Vitamin C is found in all fresh vegetables and is present in particularly large amounts in cabbage, tomatoes and citrus fruit; however, it should be noted that it is destroyed to a large extent upon cooking and storage. In animals, it is found in almost all cells, but mainly in the hypophysis (pituitary gland), in the corpus luteum and in the adrenal glands, where it protects adrenaline against a too rapid oxidation.

The functions performed by this vitamin are extremely numerous. As an oxidation-reduction agent, it plays a part in the intimate cellular metabolism and acts on the respiration and on anabolic protein processes; it is involved in the formation of ground (interstitial) substance by promoting the conversion of precollagen into collagen. In fact, it is essential in the strengthening of support tissues, such as bony and cartilagenous tissue (Negri) as well as dentin; it increases the capillary resistance (Rondoni, Hopesco, etc.) and activates numerous enzymes, such as amylase (Pure), arginose (Tauber), papain (Maschmann), cathepsin (Maver). Vitamin C has a hypoglycemic effect at high

doses (50 mg), while at lower doses it does not affect the blood sugar level (Maxia); this action is due to the fact that Vitamin C promotes hepatic glycogen fixation.

Finally, we must state that ascorbic acid has a general anti-infective action as an activator of immunity processes, in addition to an anti-bacterial action directed specifically against Staphylococcus bacteria (Kein); it increases the complementary factor and the opsonin index of serum (Nigro); it causes a more precocious (premature) and marked formation of antibodies (Dessy and Sciarra), and acts directly on the reticuloendothelial system (Malvestio). Ascorbic acid exerts a high antitoxic effect in numerous intoxications and exhibits antiallergic properties to such an extent that Bariety and Kohler were able to avoid the appearance of adrenaline pulmonary edema in a high percentage of animals by administration of vitamin C.

We have left for the end the action performed by vitamin C in the blood. In regard to hematic ions, a reduction of chloruremia has been observed, with an increase in diuresis and chloruria (Pecoraro and Comi), but with a reduction of hematic phosphorus (DeLucia and Morelli). Calcemia and potassemia are not influenced (affected) in a constant and uniform manner. Uremia and lithemia were reduced by the administration of 150 mg vitamin C on various days (Pescarmona and Quaglia). The various hematic lipid fractions have been consistently reduced (Negri and Terzi). Finally, Dogliotti, Meloni and Castellani have noted an increase of hematic glutathione. Verdollini, Cotti and Larizza have demonstrated a reduction (shortening) of the clotting time and of the dribbling (stillicidium) time.

In regard to figured elements (shaped blood elements), some disagreements have been noted; whereas Verdolini, and also DeBlasi, have not observed any noteworthy variations in the number of erythrocytes and leukocytes following intramuscular injection of vitamin C, Cislaghi and Tecilazio, after administering vitamin C by the intravenous route to nursing infants, have noted a definite increase in the number of erythrocytes, to such an extent that, according to these authors, high doses of ascorbic acid would stimulate the maturation of medulloid elements of the red series up to the point of arousing a reticulocyte crisis in peripheral blood. According to Pirastu and Lai, such a regeneration process would appear to take place also in regard to the white series, which would explain why guinea pigs treated with high doses of vitamin C appear to tolerate (endure) better a peritoneal infection. On the basis of precisely

such discrepancies and upon the advice of Prof. DiMacco, we wanted to investigate the possible effect of vitamin C, given in doses of 100 mg/kg, on red and white blood corpuscles, on the erythrosedimentation rate, on the dry residue, and on serum proteins.

Method

In carrying out our tests, we selected 4 male rabbits originating from a single breeding and kept under identical ambient conditions and a constant diet during the entire test period. From each animal blood was withdrawn at 4 different times: the first time, during fasting; the second time, 2 hours after treatment with 100 mg/kg vitamin C; the third time, 12 hours later; and the fourth time, 24 hours later.

The calculation (counting) of erythrocytes and leukocytes was done in a normal Burker chamber; the erythrosedimentation rate (ESR) was determined by the Westergreen method. In determining the dry residue (DR), a drop of blood absorbed by a small rectangular piece of absorbing paper, previously dried for at least 15 min, was weighed, and then placed into a thermostat at 110°C, leaving it there until the weight was constant. The paper was then weighed again, and variations with respect to the previous weighing were recorded; average values were calculated and always expressed as respective percentages.

Finally, to determine the various protein fractions present in the serum of the test animals, we used the paper electrophoresis method, which, although it is not a complete substitute of the classical Tiselius method, has the advantage of being more practical. The determination was carried out with a buffer solution of pH 8.6, a molecular strength of 0.1, prepared from 9.809 g sodium veronal, 7.476 g Na acetate, 60 ml of 1/10 HCl per liter, with a stabilized current of 110 v, and a duration of 12 hours.

Experimental Results

Rabbit # 1, male, 1500 g.

First Test (8 A.M., 20 Nov. 1956). Ambient temperature (at) 20°C, humidity (H) B5, barometric pressure (BP) 740, erythrocytes (E) 5,840,000, leukocytes (L) 8100, ESR 3.5, DR 10%, electrophoretic albumin ration (EAR) 50.4, globulins (G): alpha 7.7, beta 17.1, gamma 25.2.

Second test: 2 hours after 100 mg/kg vitamin C (10 AM, 20 Nov. 1956): at 21°C, H 66, BP 743, E 6,250,000, L 9700, ESR 4, DR 11%, EAR 42.7, globulins: alpha 10.5, beta 20.1, gamma 30.1.

Third test: 12 hours after 100 mg/kg vitamin C (8 PM, 20 Nov. 1956): at 22°C, H 70, BP 741, E 6,200,000, L 10,000, ESR 5, DR 10.2%, EAR 28.5, globulins: alpha 10.9, beta 20.4, gamma 40.3.

Fourth test: 24 hours after 100 mg/kg vitamin C (8 AM, 21 Nov. 1956): at 19°C, H 60, BP 740, E 5,900,000, L 10,000, ESR 3, DR 10.5%, EAR 57.2, globulins: alpha 7.2, beta 17.1.

Rabbit #2, male, 1700 g.

First test (8 AM, 20 Nov. 1956): E 5,720,000, L 8000, ESR 3, DR 12.5%, EAR 52.2, globulins: alpha 7.7, beta 12.9, gamma 30.1.

Second test: 2 hours after 100 mg/kg vitamin C (10 AM, 20 Nov. 1956): E 6,000,000, L 8500, ESR 5, DR 12%, EAR 37.2, globulins: alpha 9.8. beta 19.5, gamma 39.9.

Third test: 12 hours after 100 mg/kg vitamin C (8 PM, 20 Nov. 1956): E 6,100,000, L 8900, ESR 5, DR 11.9%, EAR 33.4, globulins: alpha 9.8, beta 19.5, gamma 39.9.

Fourth test: 24 hours after 100 mg/kg vitamin C (8 AM, 21 Nov. 1956): E 6,190,000, L 9100, ESR 3, DR 12%, EAR 51.3, globulins: alpha 7.1, beta 19.3, gamma 20.5.

Rabbit #3, male, 1300 g.

First test (8 AM, 20 Nov. 1956): E 6,200,000, L 9200, ESR 3, DR 11.9%, EAR 48.9, globulins: alpha 6.4, beta 10.9, gamma 29.5.

Second test: 2 hours after 100 mg/kg vitamin C (10 AM, 20 Nov. 1956), E 6,240,000, L 9000, ESR 4.5, DR 12%, EAR 39.1, globulins: alpha 9.2, beta 17.5, gamma 38.7.

Third test: 12 hours after 100 mg/kg vitamin C (8 PM, 20 Nov. 1956), E 6,200,000, L 9900, ESR 6, DR 12.1%, EAR 30.5, globulins: alpha 10.1, beta 18.5, gamma 43.1.

Fourth test: 24 hours after 100 mg/kg vitamin C (8 AM, 21 Nov. 1956): E 6,290,000, L 9700, ESR 3.5, DR 11.7%, EAR 59.2, globulins: alpha 9.8, beta 13.7, gamma 23.8.

Rabbit #4, male, 1600 g.

First test (8 AM, 20 Nov. 1956): E 5,800,000, L 8900, ESR 2, DR 11.7%, EAR 52.7, globulins: alpha 6.1, beta 9.8, gamma 27.8.

Second test: 2 hours after 100 mg/kg vitamin C (10 AM, 20 Nov. 1956): E 5,970,000, L 9400, ESR 6, DR 13.2%, EAR 29.9, globulins: alpha 12.5, beta 10.7, gamma 38.3.

Third test: 12 hours after 100 mg/kg vitamin C (8 PM, 20 Nov. 1956): E 6,120,000, L 10,700, ESR 6, DR 2.3%, EAR 30.1, globulins: alpha 9.5 beta 13.7, gamma 38.3.

Fourth test: 24 hours after 100 mg/kg vitamin C (8 AM, 21 Nov. 1956): E 6,200,000, L 10,900, ESR 2.5, DR 11.2%, EAR 49.5, globulins: alpha 8.6, beta 10.0, gamma 25.5.

Results and Conclusions

The data obtained in the various tests have been reported in detail above, and in order to make an interpretation and comparison of these data more easy a summary and table and corresponding diagram are given here.

Diagram 1 -

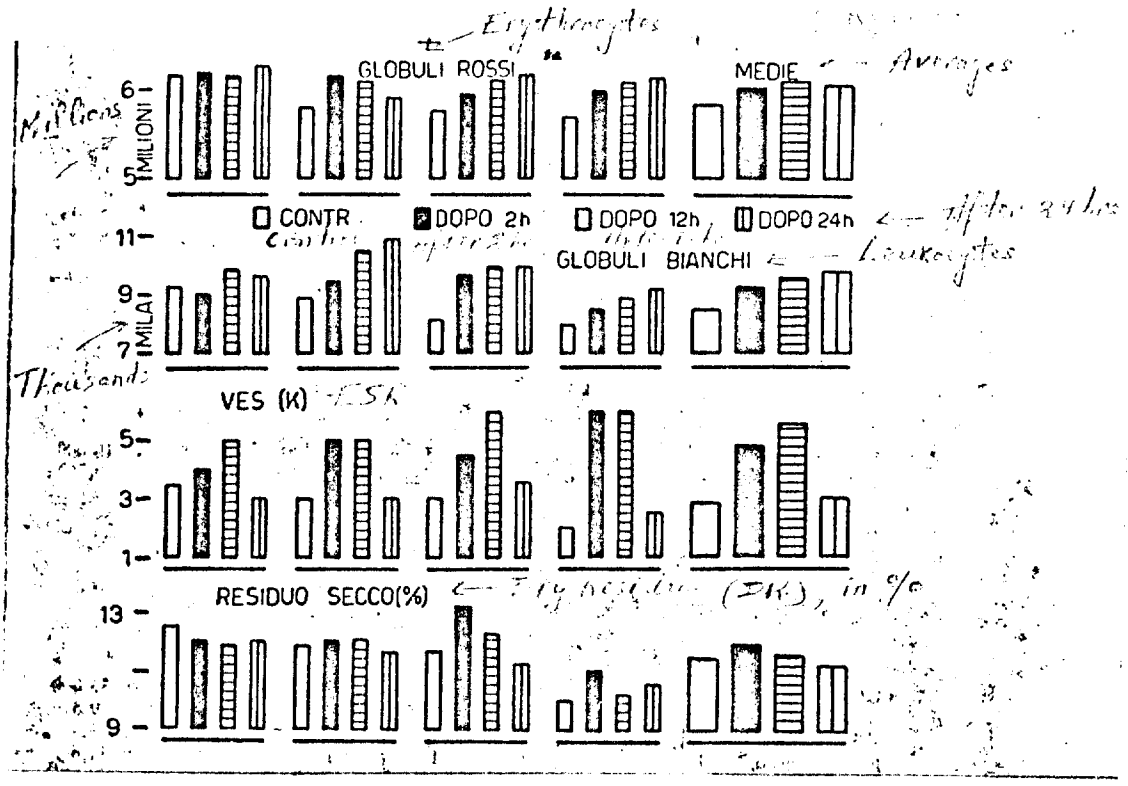

Table 1 -

Rabbit	1	2	3	4	Average values
		1st test (control)			
Erythrocytes (E)	5,840,000	5,720,000	6,200,000	5,800,000	5,890,000
Leukocytes (L)	c,100	8,000	9,200	8,900	8,500
ESR	3.5	3	3	2	2.9
Dry Residue (DR)	10	12.5	11.9	11.7	11.5
Electrophoretic albumin ratio (EAR)	50.4	51.2	48.9	52.7	50.8
Globulins - alpha	7.7	7.1	6.4	6.1	
beta	17.1	12.9	10.9	9.8	48.1
gamma	25.2	30.1	29.5	27.8	
		2nd test (100 mg/kg vitamin C)			
Erythrocytes (E)	6,250,000	6,000,000	6,240,000	5,970,000	6,115,000
Leukocytes (L)	9,700	8,500	9,000	9,400	9,150
ESR	4	5	4.5	6	4.9
Dry Residue (DR)	11	12	12	13.2	12
Electrophoretic albumin ration (EAR)	42.7	37.2	39.1	29.9	37.2
Globulins - alpha	10.5	7.9	9.2	12.5	
beta	20.1	18.5	17.5	10.7	62.3
gamma	30.1	40.1	38.7	38.3	
			3		
		3rd test (12 hours later)			
Erythrocytes (E)	6,200,000	6,100,000	6,200,000	6,120,000	6,155,000
Leukocytes (L)	10,000	8,900	9,900	10,700	9,870
ESR	5	5	6	6	5.5
Dry Residue (DR)	10.2	11.9	12.1	12.3	11.6
Electrophoretic albumin ration (EAR)	28.5	33.4	30.5	30.1	30.8
Globulins - alpha	10.9	9.8	10.1	9.5	
beta	20.4	19.5	18.5	13.7	68.8
gamma	40.3	39.9	43.1	38.3	
		4th test (24 hours later)			
Erythrocytes (E)	5,900,000	6,190,000	6,290,000	6,200,000	6,145,000
Leukocytes (L)	10,000	9,100	9,700	10,900	9,920
ESR	3	3	3.5	2.5	3
Dry Residue (DR)	10.5	12	11.7	11.2	11.3
Electrophoretic albumin ration (EAR)	57.2	51.3	59.2	49.5	45.3
Globulins - alpha	7.2	7.1	9.8	8.6	
beta	17.1	19.3	13.7	10.0	45.5
gamma	22.5	20.5	23.8	25.6	

An examination of these results discloses certain hematic variations induced by vitamin C in a 100 mg/kg intramuscular dose.

Erythrocytes appear to be little affected, although a slight but constant increase in their number is noted within 24 hours after treatment.

A slight increase in the number of leukocytes is also noted. Similarly, the dry residue (DR) exhibits slight fluctuations around normal values.

The most interesting data are noted, on the other hand, in connection with serum proteins; in fact, a definite increase of the ESR, which appears to be doubled, is noted in the second test, and above all in the third test.

At the same time, the electrophoretic diagrams show a definite increase of total globulins, and especially of the gamma-fraction, which is quite evident in the 3rd diagram. This finding can possibly be related to what has been demonstrated by Kekwick, also with rabbit serum, namely that antibodies exhibit an electrophoretic mobility corresponding to that of plasma gamma-globulins.

Thus, we can assume that the increase of the gamma fraction can be attributed to a rapid and prompt setting into circulation of antibodies, or at least of related substances. In fact, in hypervitaminosis C a superactivity of reticular cells and particularly of Kupffer cells is noted, cells which are assigned precisely to the formation of antibodies. This fact would account for the increased resistance towards infections of the organism of animals kept on a vitamin C-rich diet.

In the fourth test, finally, a return to normal of the electrophoretic diagram is noted, and the ESR is also restored to its original value.

Variations of the serum proteins are thus rapid and clearly expressed, but transient, and disappear within 24 hours after treatment.

Summary

Vitamin C, given intramuscularly to rabbits in a dose of 100 mg/kg, does not cause any appreciable changes in regard to erythrocytes and leukocytes. The dry residue of blood fluctuates around normal values.

The blood sedimentation rate is definitely higher in the second and third test; the corresponding electrophoretic diagram shows a considerable increase of globulins, and especially of the gamma fraction; 24 hours after treatment, both the ESR and the electrophoretic pattern return to their original values.

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IPERASCORBOSI E VARIAZIONI EMATICHE

UGO VOLTERRANI *GIANNI BAIOTTI*

PREMESSA

La vitamina C o acido ascorbico, isolata nel 1932 da Szent-Gyorgyi e considerata per la sua struttura chimica da Naworth e Michcel come il prodotto di ossidazione dell'esoso 1-guloso e precisamente come l'anidride dell'acido beta-chetogulonico, fu sintetizzata da Reichskin e coll., nel 1934. Attiva solo nella forma levogira (acido 1-ascorbico) si presenta come polvere cristallina bianca, facilmente solubile in H_2O, a cui impartisce netta reazione acida; stabile allo stato secco, in soluzione è facilmente ossidabile trasformandosi nell'acido deidro-ascorbico: tale proprietà caratteristica è dovuta alla presenza dei due gruppi enologi uniti da un doppio legame: la suddetta reazione è reversibile funzionando dunque tale vitamina come un sistema ossido-riduttore e come tale entra appunto nelle più svariate funzioni dell'organismo.

E' contenuta in tutti i vegetali freschi e particolarmente ricchi ne sono i cavoli, i pomodori, gli agrumi: bisogna però ricordare che con la cottura e la conservazione la vit C viene in buona parte distrutta. Negli animali si trova in quasi tutte le cellule, ma soprattutto nell'ipofisi, nel corpo luteo e nel surrene dove protegge l'adrenalina da una troppo rapida ossidazione.

Le funzioni svolte da questa vitamina sono numerosissime; come agente di ossidoriduzione entra nell'intimo metabolismo cellulare con azione sulla respirazione e sui processi anabolici proteidici: interviene nella formazione della sostanza interstiziale favorendo la trasformazione del precollageno a collageno; infatti è essenziale nel consolidamento dei tessuti di sostegno, come il tessuto osseo e cartilaginco (Negri) nonchè della dentina; aumenta la resistenza capillare (Rondoni, Lopesco, ecc.), attiva numerosi fermenti: amilasi (Pure), arginosi (Tauber), la papaina (Maschmann), la catepsina

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(Maver); ha azione ipoglicemizzante ad alte dosi (50 mg) mentre a dosi più piccole non influenza il tasso glicemico (Maxia); tale azione è dovuta al fatto che la vit C, favorisce la glicogenopessi epatica.

Dobbiamo dire infine che l'ac. ascorbico ha un'azione antinfettiva generale come attivatore dei processi immunitari oltre ad una azione antibatterica diretta specie verso lo stafilococco (Kein); aumenta il fattore complementare e l'indice opsonico del siero (Nigro); rende più precoce e marcata la formazione di anticorpi (Dessy e Sciarra) ed agisce direttamente sul sistema reticolo endoteliale (Malvestio). Ha un elevato potere antitossico in numerosi avvelenamenti ed ha proprietà antiallergiche tanto che Barièty e Kohler poterono evitare con la somministrazione di vit C, la comparsa dell'edema polmonare adrenalinico in un'alta percentuale di animali.

Abbiamo lasciato per ultimo l'azione che la vit C svolge nel sangue. A carico dei ioni ematici è stata osservata una diminuzione della cloruremia, con aumento della diuresi e della cloruria (Pecoraro e Comi); pure il fosforo (P) ematico diminuisce (De Lucia e Morelli). La calcemia e la potassiemia non sono costantemente ed uniformemente influenzate. L'uremia e l'uricemia, con la somministrazione per vari giorni di mg 150 di vit C risultarono diminuite (Pescarmona e Quaglia). Le varie frazioni lipidiche ematiche sono state costantemente diminuite (Negri e Terzi); Dogliotti e Meloni e Castellani hanno infine riscontrato un aumento del glutatione ematico. Verdolini, Cotti e Larizza hanno dimostrato una diminuzione del tempo di coagulazione e del tempo di stillicidio.

A carico degli elementi figurati si hanno alcune discordanze: mentre Verdolini in seguito a somministrazione endomuscolare di vit C non ha osservato variazioni degne di nota a carico del numero degli eritrociti e dei leucociti e così il De Blasi; Cislaghi e Tecilazio somministrandola per via endovenosa nel lattante hanno riscontrato un netto aumento degli eritrociti, tanto che secodo tali AA. le alte dosi di ac. ascorbico stimolerebbero la maturazione degli elementi midollari della serie rossa al punto da scatenare nel sangue periferico una crisi reticolocitaria. Secondo Pirastu e Lai tale processo rigenerativo avverrebbe anche a carico della serie bianca il che spiegherebbe il perchè le cavie trattate con alte dosi di vit C sembrano sopportare meglio l'infezione peritoneale. In base appunto a tali divergenze abbiamo voluto, su consiglio del prof. Di Macco inda-

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gare quale fosse l'azione della vit C, in dose di mg/kg 100 sui globuli rossi, sui globuli bianchi, sulla Velocità di Eritrosedimentazione, sul Residuo secco e sulle Proteine seriche.

TECNICA

Per l'esecuzione degli esperimenti sono stati scelti 4 conigli maschi, provenienti da un unico allevamento e tenuti per tutta la durata delle prove nelle identiche condizioni ambientali e a dieta costante. Su ogni animale sono stati eseguiti quattro prelievi: il primo a digiuno, il secondo due ore dopo il trattamento con mg/kg 100 di vit C, il terzo 12 h dopo, ed il quarto 24 h dopo.

I conteggi dei globuli rossi e dei leucociti sono stati eseguiti con la normale camera di Burker; per la velocità di Eritrosedimentazione (V.E.S.) si è seguito il metodo Westergreen; per il residuo secco (R.S.) si è pesato una goccia di sangue assorbita da un rettangolino di carta bibula preventivamente essiccata per almeno 15 min; indi si è messo il tutto in termostato a 110°, lasciandovelo fino a peso costante; poi si è ripesato registrando le variazioni rispetto alla pesata precedente e si calcola la media dei valori, esprimendoli sempre nelle rispettive percentuali.

Per determinare infine le varie frazioni proteidiche dei sieri nei vari soggetti, mi sono servito del metodo elettroforetico su carta che, pure non sostituendo completamente il classico metodo del Tiselius, offre i vantaggi di una maggiore praticità. Si è lavorato con una soluzione tampone a pH 8,6, forza molecolare 0,1, costituita in questo modo: Veronal sodico g 9,809. Acetato di sodio g 6,476. Acido cloridrico 1/10 N ml 60 per litro, con una corrente stabilizzata di 110 volt, per la durata di 12 h.

PROTOCOLLI

Coniglio 1, maschio, g 1500

I prova: (ore 8 del 20-11-56). Temp. amb. 20, umid. 65, press. bar. 740 G.R. 5.840.000; G.B. 8.100; V.E.S. 3,5, R.S. 10%; Q. elettr. Alb. 50,4; Glob. alfa 7,7, beta 17,1, gamma 25,2.

II prova: 2 h dopo mg/kg 100 vit C; ore 10 del 20-11-56. Temp. amb. 21, umid. 66, press. bar. 743. G.R. 6.250.000; G.B. 9.700; V.E.S. 4 R.S. 11%. Q. elettr. Alb. 42,7, Glob. alfa 10,5, beta 20,1, gamma 30,1.

III prova: 12 h dopo mg/kg 100 vit C, ore 20 del 20-11-56.

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Temp. amb. 22, umid. 70, press. bar. 741. G.R. 6.200.000; G.B. 10.000; V.E.S. 5, R.S. 10,2%. Q. elettr. Alb. 28,5; Glob. alfa 10,9, beta 20,4, gamma 40,3.

IV prova: 24 h dopo mg/kg 100 vit C ore 8 del 21-11-56. Temp. amb. 19, umid. 60, press. bar. 740. G.R. 5.900.000; G. B. 10.000; V.E.S. 3; R.S. 10,5%. Q. elettr. Alb. 57,2, Glob. alfa 7,2, beta 17,1, gamma 22,5.

Coniglio 2 maschio, g 1700

I prova: ore 8 del 20-11-56. G. R. 5.720.000; G.B. 8.000; V.E.S. 3, R.S. 12,5%. Q elettr. Alb. 52,2, Glob. alfa 7,7, beta 12,9, gamma 30,1.

II prova: 2 h dopo mg/kg 100 vit C, ore 10 del 20-11-56. G.R. 6.000.000; G. B. 8.500 V.E.S. 5, R.S. 12%. Q. elettr. Alb. 37,2, Glob. alfa 7,9, beta 18,5, gamma 40,1.

III prova: 12 dopo mg/kg 100 vit C, ore 20 del 20-11-56 G. R. 6.100.000; G.B. 8.900; V.E.S. 5; R.S. 11,9%. Q. elettr. Alb. 33,4; Glob. alfa 9,8, beta 19,5, gamma 39,9.

IV prova: 24 h dopo mg/kg 100 vit C, ore 8 del 21-11-56. G.R. 6.190.000; G.B. 9,100; V.E.S. 3, R.S. 12%. Q. elettr. Alb. 51,3; Glob. alfa 7,1; beta 19,3, gamma 20,5.

Coniglio 3, maschio, g 1300

I prova: ore 8 del 20-11-56. G.R. 6.200.000; G.B. 9.200; V.E.S. 3; R.S. 11,9%. Q. elettrof. Alb. 48,9. Glob. alfa 6,4, beta 10,9, gamma 29,5.

II prova: 2 h dopo mg/kg 100 vit C, ore 10 del 20-11-56. G.R. 6.240.000; G.B. 9.000; V.E.S. 4,5; R.S. 12%. Q. elettrof. Alb. 39,1. Glob. alfa 9,2, beta 17,5, gamma 38,7.

III prova: 12 h dopo mg/kg 100 vit C. ore 20 del 20-11-56. G.R. 6.200.000; G.B. 9.900; V.E.S. 6, R.S. 12,1%. Q. elettrof. Alb. 30,5, Glob alfa 10,1, beta 18,5, gamma 43,1.

IV prova: 24 h dopo mg/kg 100 vit C. ore 8 del 21-11-56. G.R. 6.290.000; G.B. 9.700; V.E.S. 3,5; R.S. 11,7%. Q. elettr. Alb. 59,2, Glob alfa 9,8, beta 13.7, gamma 23,8.

Coniglio 4 maschio, g 1600

I prova: ore 8 del 20-11-56. G.R. 5.800.000; G.B. 8.900; V.E.S. 2, R.S. 11,7%. Q. elettr. Alb. 52,7; Alb. alfa 6,1, beta 9,8, gamma 27,8.

II prova: 2 h dopo mg/kg 100 vit C. ore 10 del 20-11-56. G.R. 5.970.000 G.B. 9.400; V.E.S. 6, R.S. 13,2%. Q. elettrof. Alb. 29,9; Glob. alfa 12,5, beta 10,7, gamma 38,3.

III prova: 12 h dopo mg/kg 100 vit C. ore 20 del 20-11-56. G.R. 6.120.000; G.B. 10.700; V.E.S. 6, R.S. 2,3. Q. elettrof. Alb. 30,1; Glob. alfa 9,5, beta 13,7, gamma 38,3.

IV prova: 24 h dopo mg/kg 100 di vit C. ore 8 del 21-11-56. G.R. 6.200.000; G.B. 10.900; V.E.S. 2,5; R.S. 11,2%. Q. elettr. Alb. 42,5, Glob. alfa 8,6, beta 10,6; gamma 25,5.

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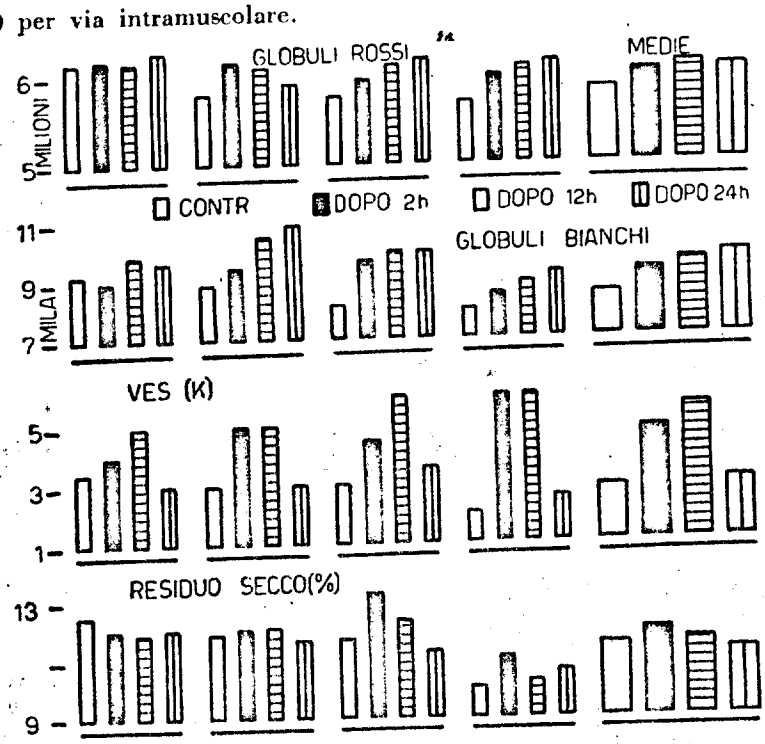
RISULTATI E CONCLUSIONI

I dati ottenuti nelle varie prove sono stati riportati per esteso nei protocolli e per renderne più agevole la lettura ed i confronti alleghiamo le tabelle riassuntive con i relativi grafici.

Dall'esame di questi risultati possiamo mettere in evidenza alcune variazioni ematiche provocate dalla vit C alla dose di mg/kg 100 per via intramuscolare.



Gli *eritrociti* appaiono poco influenzati notandosi appena un sia pur costante ma lieve aumento nelle 24 h successive al trattamento.

Così anche i *leucociti* tendono leggermente ad aumentare. Analogamente il R.S. (residuo secco) subisce delle leggere oscillazioni attorno ai valori normali.

I dati più interessanti si hanno invece a carico dei sieroproteidi: al II e soprattutto al III saggio si nota infatti un netto aumento della V.E.S. che appare raddoppiata.

Contemporaneamente i tracciati elettroforetici ci dimostrano un netto aumento della globuline in toto ma specialmente della frazio-

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ne gamma che nel III tracciato risulta molto evidente. Questo reperto si potrebbe mettere in relazione con quanto è stato dimostrato, proprio sul siero del coniglio, dal Kekwick e cioè che gli anticorpi presentano una mobilità elettroforetica corrispondente a quella delle gamma-globuline del plasma.

Potremmo pertanto pensare che l'aumento della frazione gamma sia riconducibile ad una messa in circolo, rapida e pronta di anti-

TABELLA I

Coniglio			3	4	Media
	I Saggio (Controllo)				
Globuli Rossi	5.840.000	5.720.000	6.200.000	5.800.000	5.890.000
Globuli bianchi	c.100	8.000	9.200	8.900	8.500
V.E.S. (k)	3,5	3	3	2	2,9
Residuo secco (%)	10	12,5	11,9	11,7	11,5
Q. elettroforetico Alb.	50,4	51,2	48,9	52,7	50,8
Glob. alfa	7,7	7,1	6,4	6,1	
Glob. beta	17,1	12,9	10,9	9,8	48,1
Glob. gamma	25,2	30,1	29,5	27,8	
	II Saggio (Vit. C mg/Kg 100)				
Globuli Rossi	6.250.000	6.000.000	6.240.000	5.970.000	6.115.000
Globuli bianchi	9.700	8.500	9.000	9.400	9.150
V.E.S. (k)	4	5	4,5	6	4,9
Residuo secco (%)	11	12	12	13,2	12
Q. elettroforetico Alb.	42,7	37,2	39,1	29,9	37,2
Glob. alfa	10,5	7,9	9,2	12,5	
Glob. beta	20,1	18,5	17,5	10,7	62,3
Glob. gamma	30,1	40,1	38,7	38,3	
	III Saggio (12 h dopo)				
Globuli Rossi	6.200.000	6.100.000	6.200.000	6.120.000	6.155.000
Globuli bianchi	10.000	8.900	9.900	10.700	9.870
V.E.S. (k)	5	5	6	6	5,5
Residuo secco (%)	10,2	11,9	12,1	12,3	11,6
Q. elettroforetico Alb.	28,5	33,4	30,5	30,1	30,8
Glob. alfa	10,9	9,8	10,1	9,5	
Glob. beta	20,4	19,5	18,5	13,7	68,8
Glob. gamma	40,3	39,9	43,1	38,3	
	IV Saggio (24 h dopo)				
Globuli Rossi	5.900.000	6.100.000	6.290.000	6.200.000	6.145.000
Globuli bianchi	10.000	9.100	9.700	10.900	9.920
V.E.S. (k)	3	3	3,5	2,5	3
Residuo secco (%)	10,5	12	11,7	11,2	11,3
Q. elettroforetico Alb.	57,2	51,3	59,2	49,5	45,3
Glob. alfa	7,2	7,1	9,8	8,6	
Glob. beta	17,1	19,3	13,7	10,0	45,5
Glob. gamma	29,5	20,5	23,8	25,6	

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corpi o per lo meno di sostanze affini. Si nota infatti, nell'iperascorbosi, una iperattività delle cellule del reticolo e particolarmente delle cellule del Kupffer, cellule deputate appunto alla formazione di anticorpi. Questo ci renderebbe conto dell'aumentata resistenza dell'organismo di animali in dieta ricca di Vit C di fronte alle infezioni.

Al IV prelievo infine si nota un ritorno alla norma del tracciato elettroforetico. Così pure la V.E.S. è ricondotta al valore di partenza.

Le variazioni dei sieroproteidi sono quindi pronte e molto marcate, ma fugaci, esaurendosi nello spazio di 24 h dal trattamento.

RIASSUNTO

La vit C, alla dose di mg/kg 100, per via intramuscolare, nei conigli, non provoca variazioni degne di nota per quanto riguarda gli eritrociti e i leucociti. Il residuo secco del sangue oscilla attorno ai valori normali. La velocità di emosedimentazione è nettamente aumentata al 2° e al 3° saggio: corrispondente il tracciato elettroforetico presenta un aumento considerevole delle globuline e soprattutto della frazione gamma. Dopo 24h dal trattamento sia la VES che il quadro elettroforetico son tornati ai valori primitivi.

RÈSUMÈ. La vit. C., à la dose de 100 mg/Kg, par voie intramusculaire chez les lapins, ne provoque pas des variations dignes de considération pour ce qui concerne les érythrocytes et les leucocytes. Les résidu sec du sang est presque normal. La vitesse de sédimentation du sang est nettement augmentée à la 2e et à la 3e épreuve: correspondement, la courbe électrophorétique présente une augmentation considérable des globulines, et surtout de la fraction gamma. 24 heures aprés le début du traitment, soit la vitesse de sédimentation du sang que le cadre électrophorétique se sont rétablis aux valeurs initielles.

SUMMARY. In rabbits, C-vitamin (intramuscularly, 100 mg/Kg) does not cause appreciable changes as to erythrocytes and leucocytes. The dry residuum of the blood is more or less normal. The blood-cell sedimentation rate shows at the 2nd and 3rd test, an evident increase; correspondingly the electrophoretic curve exhibits a considerable globuline increase, specially of their gamma fraction. 24 hours after onset of the treatment, as well blood-cell sedimentation rate as electrophoretic picture are normal again.

ZUSAMMENFASSUNG. Bei Kaninchen, ruft C-Vitamin (intramuskulär, 100 Mg/Kg) keine bedeutenden Veränderungen was Erythrocyten und Leukocyten betrifft, hervor. Der trockenrückstand des Bluteszeigt etwa normale Werte. Die Blutkörperchen Senkungsgeschwindigkeit

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wird am 2.en u.3en Teste deutlich erhöht; die elektro phjoretische Kurve zeigt entsprechend eine ausgesprochene Erhöhung der Globuline, besonders ihrer Gammafraktion. 24 Stunden nach Anfang der Behandlung haben sowohl die Blutkörperchen Senkungsgeschwindigkeit als auch das elektrophoretische Bild die Ausgungswerte wiedererreicht.

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DISORIENTATION AFTER THE FIRST CONFINEMENT AND VITAMIN C

by Dr. P.A. Voute, The Hague

(Summary [Samenvatting] not translated since there is an English summary at the end of the article.)

Every pediatrician who works for some time at a consultation bureau must have seen mothers who suffer from "disorientation." The experienced personnel in charge of a consultation bureau recognize these women promptly by the set expression of their faces and by the absence of laughter which, in other mothers, appears of itself on their faces when they speak about their young babies. It is as if they have to endure some great sorrow.

At the first visit to the consultation bureau -- often in the first month after confinement -- the disorientation is at its zenith. I have never seen it develop later in the year of supervision (control year).

If we ask the mother whether "everything has changed" and whether everything is different from what she had thought, then one sees anxious eyes in which the tears appear with a seeking for help, and hesitatingly the question is confirmed.

There is about the woman a kind of desperation, she doesn't understand herself, everything has become different. She has lost contact with her environment and with her child. Even a cordial, affectionate husband cannot do any good; many of these women also still feel guilty because they have had no fortunate experience, while they are considered fortunate and they pretend that they are so, still their laughter is only a shadow of a laugh.

Whenever one tells them that the condition frequently occurs after the birth of the first child and is caused by confinement and by delivery and that they are not alone in the condition but many women experience it completely and that it passes, these words give them relief from tension which is expressed almost always in weeping. ("There is safety in numbers" [English in text], says Emerson in his essay on self-reliance and this is a true statement in the case of the experience of anxiety; it indicates that loneliness is a cause of anxiety and that something of security returns whenever one knows that one is not alone in his suffering).

These women experience a disorientation from their environment, from their rooms of their houses, from individuals well known to them and from their child; they feel that they have become something different; they are alienated from themselves, they do not recognize themselves and are in a quiet panic as a result; they see the ground gone from under their feet. A calming word "that they are not alone" brings release of tension, but in no way removes this condition. The assurance "that it passes" is thankfully accepted but only half-believed.

It is typical that they never complain spontaneously, they speak very little or not at all, their attitude is one of waiting. Over-sensitiveness, rapidity of feeling, unusual emotionality are lacking. The look in their eyes is perhaps somewhat fixed, but certainly anxious. Indications of a psychosis or of a neurosis

even of a hyperesthetic-emotional complex of symptoms are not merely absent, the disorientation rather blocks off the usual emotional life and the variations of it. It is as if the women temporarily live behind glass, experience this unconsciously and and go on automatically with their existence without really participating in it.

I am accustomed to recommend these patients to the special attention of the visiting physician (making house calls); at the beginning I have them return once a week and treat them with restoration of comprehension and with the suggestion of improvement that will come about quickly. But I experienced each time the oppressive feeling that they are psychically in danger, that a psychosis is pending. Almost always the condition resolves itself gradually and after three to four months the women are "normal", although they are somewhat perplexed? and can remain turned in on themselves and can continue to remember the first months in which they should have been happy but were nevertheless unhappy.

In the course of the thirty years in which I have acted as chief of a consultation bureau, I have seen this condition of "disorientation" again and again and it is my conviction that it must be commonly recognized by pediatricians. Its frequency is greater than one would think and in greater or lesser degree, I estimate that one out of fifteen to twenty mothers has experienced something of this disorientation after the first confinement.

I do not know whether the situation as described is recognized and named as such in the literature but it is commonly recognized by experienced laymen. In the book Becoming A Mother (Moeder worden) which is distributed by the Zwitsal factory (and is a translation of Your Baby and You by Dr. W. deKok), one finds on pages 76-78 a clear description of the dissatisfaction of the first weeks after confinement, characterized in the book as "tearfulness, despondency, a dead-tired feeling" etc., accompanied by the very correct reassurance that it will pass. This dissatisfaction has however a characteristic of its own here designated as "disorientation."

The "disorientation" occurs in moderate and serious form; whenever the physician is hurried, the consultation bureau gets through quickly , begins to ask how many mothers it has to supervise and is preoccupied by what the day must bring, the physician or the staff of the consultation bureau has a good chance not to recognize the overall picture. It is necessary that one listen with an open heart. Almost always the child of such a mother reacts by crying a lot and by being unhappy, which makes the situation worse, for the well-fed, mobile, happy smile of a young baby is an experience that has a curative effect; this is withheld from these women, even the child is unhappy. It is not a question of whether unwed mothers experience disorientation to a greater degree. This disorientation has nothing to do with the attitude toward the child.

I have always had my consultation bureau among the simplest people, in Utrecht, Amsterdam, and at The Hague, but in my opinion disorientation has no connection with malnutrition or impoverished circumstances. I have had the impression that in the case of the most careful mothers, who look forward tensely to the arrival of their first child, the condition of disorientation more easily developed.

A connection with breast-feeding is absent, the condition is not made worse by breast-feeding. It is indeed my impression that in the case of fully developed disorientation, there is very little breast-feeding, probably because extra feeding is offered quickly to the child who cries a lot.

According to my impression, I have noticed disorientation after the birth of the first child.

I encountered a serious case in April, 1962. It concerned a woman who came to the consultation bureau with a six-week old child and who was only half amenable to the treatment by suggestion indicated above. She first of all did not believe it and was quite anxious about the inner change in herself by which happiness and joy had disappeared.

Just at that time I read in this Journal the article by DeSmit and DeWaart (1962) about Amentia Puerperalis, treated with vitamin C. The authors there describe the picture of disorientation in its extreme form, but what they write is valid in a lesser degree but entirely similar for the above indicated symptom complex.

On the basis of the article mentioned, I advised this woman to use vitamin C, ten 50 mg. tablets. When she returned after one week, I listened sceptically; the improvement was unmistakeable, the nurse also perceived this immediately. I carefully asked information about how her husband, her mother-in-law, and the other people of her environment found her. The reports were of the same tenor. The contact with her environment was restored without anything remaining except a memory. The recovery continued (i.e., there was no relapse); the woman was a "normal" mother with a child that reacted happily and thereby helped her to forget the previous phase.

Apart from that, the suffering experienced during the first months was so deeply impressed on this woman that she was not willing to accept a second pregnancy because of fear of a repetition. What had occurred during the first weeks around her, to her, and in her remained for her a picture of terror. This is noteworthy because the disorientation, as it slowly passes toward normal existence is obviously more naturally accepted.

In a letter to the personal (visiting) physician, I described her condition, and I advised the woman to speak to him; she has an understanding physician who is open to the suggestion of another. (Alas this is not always the case; personal physicians are, vis-à-vis the chiefs of consultation bureau incomprehensively negative, and in too many cases a suggestion by the consultation bureau is rejected). The anxiety was so profound in this woman that she

did not go to her personal physician; when her child was a year old she still had my letter in her possession.

Another woman with a clearly indicated disorientation, though in a lesser degree, returned after a week of using vitamin C for checking and answered my question as to how she was: "I am able to cope with everything much better and I feel much calmer."

A third woman in the same circumstances answered after a week of using vitamin C: "I take more pleasure in living and am more cheerful in my work." To my question as to when she had noticed the change, the answer was: "After three or four days; before that I didn't notice any change."

There is also something objective in this change, the women are more lively, their eyes are more mobile. It is seen from their whole bearing that they are again living with and from an accustomed reserve.

It is my conviction that these young mothers with disorientation are not proper patients for a neurologist. A true psychosis will be perceived from the disposition or by the environment. One does these women individually or as a group a service by not making them "patients". The physician who wishes to know more of what is happening in them, who wishes to dig deeper, must not surrender to this desire for thereby the misery is driven in further. The situation should remain within the household. It is thus indicated that the responsibility should be accepted and this is the more

permissible because the symptoms of disorientation are always resolved by themselves as a temporary phase, ending speedily.

In front of the consultation bureau we have placed a pound of vitamin C in 50 mg. tablets and at present we give the women who notice them a bag of vitamin C to take along.

My experience is slight but I am firmly convinced:

1. that amentia puerperalis, which was described by DeSmit and DeWaart in its fully developed form, occurs much more often in more moderate forms than is recognized;

2. that this condition arrises after confinement and is not already present during pregnancy;

3. that the condition is caused by confinement and delivery;

4. that the rapid origination of disorientation and the gradual recovery from it form a parallel with a cause of trauma;

5. that a predisposition of the individual constitution is necessary for the development of disorientation;

6. that the chief symptom is disorientation and that this, according to which the woman no longer recognizes herself, can lead to panic;

7. that other psychic symptoms such as hallucinations or true peculiar feelings or abnormal emotionality are absent;

8. that one woman with serious disorientation and two women with more moderate forms reacted excellently to treatment with vitamin C;

9. that it is recommended that this harmless therapy be tested further, in which it is perhaps not necessary to use large doses of vitamin C.

10. that one must be attentive to the development of disorientation in the cases of lying-in women and mothers who bring their infants for checking;

11. that it ought to be done on an individual basis and that it is foolish to overload all lying-in woman with vitamin C.

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Vervreemding na eerste bevalling en vitamine C

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Samenvatting:

De auteur beschrijft een toestand van vervreemding die bij vrouwen kort na de geboorte, vooral van het eerste kind, tot ontwikkeling kan komen: hij beschouwt, in navolging van N. W. DE SMIT en C. DE WAART (1962), dergelijke toestanden als lichte vormen van amentia puerperalis. Toediening van vitamine C werkt gunstig.

Iedere kinderarts die enige tijd op een consultatiebureau werkt, moet moeders hebben gezien die aan „vervreemding" leden. De ervaren leider van een consultatiebureau herkent deze vrouwen prompt aan een strakke uitdrukking van het gelaat en aan het ontbreken van een lach die bij andere moeders vanzelf op het gelaat verschijnt als zij over de jonge zuigeling spreken. Het is of zij een groot verdriet te verduren hebben.

Bij het eerste bezoek aan het consultatiebureau — veelal in de eerste maand na de bevalling — is de vervreemding op haar hoogtepunt. Nooit zag ik haar later in het controlejaar tot ontwikkeling komen.

Vragen wij de moeder of het „alles anders is geworden" en alles anders is dan zij gedacht had, dan ontmoet men angstige ogen waarin de tranen opwellen, met een verzoek om hulp, en aarzelend wordt die vraag bevestigd.

Er is bij de vrouw een radeloosheid, zij begrijpt zichzelve niet, alles is anders geworden. Zij heeft het contact met haar omgeving en met het kind verloren. Ook een hartelijke, liefhebbende man kan er geen goed aan doen: vele van deze vrouwen voelen zich nog schuldig ook, omdat zij geen geluksbelevenis hebben, terwijl zij toch geacht worden nu gelukkig te zijn en zij veinzen het te zijn, doch hun lach is slechts een schaduw van een lach.

Wanneer men hun zegt dat die toestand veel voorkomt na de geboorte van het eerste kind en veroorzaakt wordt door de bevalling en door de ontzwangering en dat zij niet alleen staan maar vele vrouwen geheel hetzelfde ervaren en dat het overgaat, geven die woorden haar een ontspanning, die zich vrijwel altijd in huilen ontlaadt. („There is safety in numbers", zegt EMERSON in zijn *Essay on self-reliance* en dit is een waar woord bij angstbelevenis; het geeft aan dat de eenzaamheid beangstigend is en dat er iets van veiligheid terugkomt wanneer men weet dat men niet alléen is in zijn ellende).

De vrouwen ervaren een vervreemding van hun omgeving, van hun huiskamer, van de hun welbekende personen en van hun kind; zij voelen dat zij anders zijn geworden; zij zijn van zichzelf vervreemd, zij herkennen zichzelve niet en zijn in een stille paniek daarover; zij zijn de grond onder hun voeten kwijt. Een geruststellend woord „dat zij niet alleen staan" geeft ontspanning, maar heft deze toestand in genen dele op. De verzekering „dat het over gaat" wordt dankbaar aanvaard, doch maar half geloofd.

Typerend is, dat zij nooit spontaan klagen, zij spreken weinig of niet, hun houding is afwachtend. Overgevoeligheid, snel geroerd zijn, ongewone emotionaliteit ontbreken. De blik van de ogen is misschien wat star, maar zeker angstig. Aanwijzingen voor een psychose of een neurose, ook van het hyperesthetisch-emotionele complex van symptomen zijn niet slechts afwezig, de vervreemding blokkeert veeleer het gewone gevoelsleven en de varianten daarvan. Het is alsof die vrouwen tijdelijk achter glas leven, dit zelf onbewust ervaren en automatisch met hun bestaan doorgaan zonder waarachtig daaraan deel te hebben.

Ik ben gewoon deze patiënten in de speciale aandacht van de huisbezoekster aan te bevelen; aanvan-

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kelijk laat ik ze eens in de week terugkomen en behandel ze met het bijbrengen van begrip en met suggestie van spoedig komende beterschap. Maar ik ervaar telkens weer het benauwende gevoel dat zij psychisch in gevaar zijn, dat er een psychose hangende is. Vrijwel steeds lost de toestand geleidelijk aan op, en na drie tot vier maanden zijn de vrouwen „normaal", ofschoon zij wat verstild en naar binnen gekeerd kunnen blijven en zich die eerste maanden, waarin zij gelukkig moesten zijn doch ongelukkig waren, kunnen blijven herinneren.

In de loop van de dertig jaren waarin ik als leider van een consultatiebureau optreed, heb ik deze toestand van „vervreemding" steeds weer gezien en het is mijn overtuiging dat die bij de kinderartsen algemeen bekend moet zijn. De frequentie is hoger dan men zou denken en in meer of minder duidelijke mate schat ik dat een van de vijftien à twintig moeders iets van deze vervreemding na haar eerste bevalling heeft ervaren.

Ik weet niet of de beschreven situatie als zodanig in de literatuur bekend en benaamd is, maar ze is bij ervaren leken algemeen bekend. In het boek *Moeder worden* dat door de Zwitsalfabriek wordt verspreid (en een vertaling is van Mrs. Dr. W. DE KOK: *Your baby and you*) vindt men op bl. 76-78 een duidelijke beschrijving over de ontstemming der eerste weken na de bevalling, daar gekenmerkt met „huilerigheid, mismoedigheid, doodmoe-voelen" enz., begeleid van de zeer juiste geruststelling dat het overgaat. Deze ontstemming heeft echter een zeer eigen, hier met „vervreemding" aangegeven karakter.

De „vervreemding" komt in lichte en ernstige vorm voor; wanneer de arts gejaagd is, het consultatiebureau snel afwerkt, begint met te vragen hoeveel moeders er wachten en gepreoccupeerd is door wat er die dag moet gebeuren, maakt hij of zij een goede kans het beeld niet te herkennen. Het is nodig dat men met een open hart luistert. Vrijwel altijd reageert het kind van zo'n moeder door veel te huilen en ongelukkig te zijn, hetgeen de situatie verergert, want van een welgedaan, gezapig, gelukkig glimlachje van een jonge baby gaat nu eenmaal een belevenis uit die genezend werkt; dit wordt deze vrouwen onthouden, ook het kind is ongelukkig. Er is geen sprake van dat ongehuwde moeders in hoger frequentie de vervreemding tonen. Met de instelling tegenover het kind heeft deze vervreemding niets te maken.

Ik heb mijn consultatiebureau altijd onder de eenvoudigste bevolking gehad, zowel in Utrecht, Amsterdam als in Den Haag, maar ook met ondervoeding of armelijke omstandigheden hangt de vervreemding mijns inziens niet samen. Zelfs heb ik wel eens de indruk gehad dat juist bij uiterst zorgzame moeders, die met een gespannen verwachting de komst van hun eerste kind tegemoetzagen, de toestand van vervreemding gemakkelijker tot ontwikkeling kwam.

Een samenhang met de borstvoeding ontbreekt, de toestand wordt niet verergerd door de borstvoeding. Wel is het mijn indruk dat er, bij vol ontwikkelde vervreemding, van de borstvoeding niet veel terechtkomt, waarschijnlijk doordat aan het veel huilende kind spoedig bijvoeding wordt geboden.

Naar mijn indruk heb ik de vervreemding vooral waargenomen na de geboorte van het eerste kind.

Een ernstig geval nam ik in april 1962 waar. Het betrof een vrouw die op het consultatiebureau kwam met een kind van zes weken en die voor de hierboven aangegeven suggestiebehandeling zelfs maar half toegankelijk was. Zij geloofde het eerst niet en was heel angstig voor de innerlijke verandering van haarzelve waardoor geluk en vreugde waren geweken.

Juist in die tijd las ik in dit *Tijdschrift* het artikel van DE SMIT en DE WAART (1962) over amentia puerperalis, behandeld met vitamine C. De auteurs beschrijven daar het beeld van de vervreemding in zijn extreme vorm, maar wat zij schrijven geldt in verkleinde mate, doch geheel gelijk voor het hierboven aangegeven symptomencomplex.

Op grond van genoemde publikatie heb ik deze vrouw geraden, vitamine C te gebruiken, tien tabletten van 50 mg. Toen zij na een week terugkwam heb ik sceptisch geluisterd; de beterschap was onmiskenbaar, ook de verpleegster nam deze terstond waar. Ik heb voorzichtig geïnformeerd wat haar man, wat haar schoonmoeder en haar verdere omgeving van haar vonden. De berichten waren eensluidend. Het contact met haar omgeving was hersteld zonder andere resten na te laten dan een herinnering. De genezing is gebleven; de vrouw was een „normale" moeder met een kind dat gelukkig ging reageren en daardoor meehielp de vorige fase te doen vergeten.

Overigens is de doorgemaakte ellende van de eerste maanden zo diep ingesneden bij deze vrouw dat zij geen tweede zwangerschap aandurft uit angst voor een herhaling. Wat zich in die eerste weken om haar, aan haar en in haar voltrok, is voor haar een schrikbeeld gebleven. Dit is merkwaardig, omdat de vervreemding, als ze langzaam overgaat naar het normale bestaan kennelijk natuurlijker wordt aanvaard.

In een brief aan de huisarts legde ik de stand van zaken uit, en ik heb de vrouw geraden, met hem te spreken; zij heeft een begrijpende huisarts, die open staat voor een suggestie van een ander. (Helaas is dat niet altijd het geval; tegenover leiders van consultatiebureaus staan huisartsen onbegrijpelijk negatief en in een te hoge frequentie wordt een suggestie van het consultatiebureau afgewezen). De angst zit deze vrouw echter zo diep dat zij niet naar haar huisarts is gegaan; toen haar kind een jaar was, had zij mijn brief nog bij zich.

Een andere vrouw met duidelijke vervreemding, zij het in lichter mate, kwam na een week vitamine-C-gebruik terug ter controle en antwoordde op de vraag hoe het ging: „ik kan het alles veel beter aan en ik voel mij veel rustiger".

Een derde vrouw onder dezelfde omstandigheden antwoordde, na een week vitamine-C-gebruik: „ik heb er meer aardigheid in gekregen en doe het werk met meer plezier". Op mijn vraag wanneer zij die verandering had waargenomen luidde het antwoord: „na 3 of 4 dagen; eerst merkte ik er niets van".

Ook objectief is er iets veranderd, de vrouwen zijn levendiger, de ogen zijn beweeglijker. Uit hun hele gedrag blijkt dat zij weer met en uit een gewone reserve leven.

Het is mijn overtuiging dat deze jonge moeders met vervreemding geen klanten voor de zenuwarts zijn. Een echte psychose zal uit den aard ook door de om-

geving onderkend worden. Men bewijst deze vrouwen persoonlijk en maatschappelijk een dienst door er geen „patiënten" van te maken. De arts die meer wil weten van wat er in haar omgaat, die dieper wil graven, moet aan dit verlangen niet toegeven, want daardoor grift de misère zich in. De situatie dient in het gewone huiselijke vlak te blijven. Het is dus aangewezen hier zelf de verantwoordelijkheid te aanvaarden en dit is te meer geoorloofd omdat de verschijnselen van vervreemding als een tijdelijke fase op korte termijn steeds vanzelf oplossen.

Voor het consultatiebureau hebben wij een pond vitamine C in tabletjes van 50 mg laten komen en wij geven tegenwoordig de vrouwen die ervoor in aanmerking komen, een zakje vitamine C mee.

Mijn ervaring is klein maar het staat voor mij vast:

1. dat de amentia puerperalis, die door De Smit en De Waart in haar vol ontwikkelde vorm werd beschreven, in lichtere vormen veel meer voorkomt dan bekend is;

2. dat deze toestand ontstaat na de bevalling en niet in de zwangerschap reeds aanwezig is;

3. dat de toestand veroorzaakt wordt door bevalling en ontzwangering;

4. dat het in korte tijd ontstaan van vervreemding en de geleidelijke genezing daarvan een parallel met een traumatische oorsprong toestaan;

5. dat een gestelspredispositie noodzakelijk is voor de ontwikkeling van de vervreemding;

6. dat het hoofdsymptoom vervreemding is en dat deze, doordat de vrouw zichzelve niet meer herkent, tot paniek kan voeren;

7. dat andere psychische verschijnselen zoals hallucinaties of echte zondegevoelens of abnormale emotionaliteit ontbreken;

8. dat één vrouw met ernstige vervreemding en twee vrouwen met lichtere vormen op toediening van vitamine C uitstekend reageerden;

9. dat het aanbeveling verdient, deze onschuldige therapie verder te proberen, waarbij het misschien niet nodig is, grote doses vitamine C te gebruiken;

10. dat men bij kraamvrouwen en bij moeders die hun zuigelingen voor controle brengen, bedacht moet zijn op de ontwikkeling van de vervreemding;

11. dat er geïndividualiseerd dient te worden en dat het zinloos is, alle kraamvrouwen met vitamine C te overladen.

Summary:

Estrangement after the first partus and vitamin C. - Estrangement from the well-known environment: persons, family, daily surroundings, the home, the child, the own personality is observed by the author as a common mental state of mothers during the first months after giving birth especially of the first child. The author is convinced that this loss of contact is a modified, light form of amentia puerperalis; following a publication of N. W. de Smit and C. de Waart (1962) he suggests treatment with acidum ascorbinicum and he describes a case of serious estrangement and two more lenient ones of quick and complete cure under this treatment.

Literatuur:

Smit, W. N. de en C. de Waart (1962) Relatie tussen amentiele psychose in het puerperium en het ascorbinezuurgehalte in het plasma. *Ned. T. Geneesk.* **106**, 159.

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Effects of Ascorbic Acid Deficiency on Kinetics of Drug Hydroxylation in Male Guinea Pigs

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Abstract ☐ The effects of ascorbic acid (vitamin C) deficiency on components of drug-hydroxylating systems in guinea pig liver were investigated. Although the liver weight–body weight ratio was increased, the concentration of microsomal protein was markedly less in ascorbic acid-deficient guinea pigs. This was reflected in a decrease in aniline and hexobarbital hydroxylation reactions when calculated on a unit of liver weight; however, when analyzed per unit of microsomal protein, ethylmorphine demethylase activity was unaffected. The K_m's for these substrates, as well as the ethyl isocyanide difference spectra, were unchanged, indicating that no qualitative changes had occurred in the enzymes responsible for their metabolism or in the cytochrome P-450. Aniline metabolism per unit protein was depressed by ascorbic acid deficiency, as was the content of cytochromes P-450 and b_5. The return of function by a single injection of ascorbic acid given 1–24 hr. prior to decapitation was not frequently observed. Induction with sodium phenobarbital was not blocked by this dietary deficiency state.

Keyphrases ☐ Ascorbic acid deficiency—effect on kinetics of drug hydroxylation, guinea pig liver ☐ Drug hydroxylation kinetics—effect of ascorbic acid deficiency, guinea pig liver

The duration and intensity of action of many drugs are largely determined by the speed at which they are metabolized in the body (1). The rate of such drug metabolism may be altered by drug pretreatment, hormones, or the nutritional status of the animal. Increased rates appear to be due to an increased concentration of microsomal enzyme or to the increased affinity of the enzyme for the substrate. Past studies

Table I—Effect of Ascorbic Acid Deficiency on Body Weight, Liver Weight, and Hepatic Microsomal Protein Content of Guinea Pigs

	Days on Diet	Control	Ascorbic Acid Deficiency	p
Terminal body weight, g. ± *SE*	12	340.1 ± 15.6 (15)	281.8 ± 11.6 (15)	<0.01
	18	389.3 ± 6.8 (4)	302.2 ± 20.8 (4)	<0.05
Liver weight g. ± *SE*	12	13.13 ± 0.79 (15)	13.13 ± 0.59 (15)	>0.05
	18	13.95 ± 1.24 (4)	13.40 ± 1.32 (4)	>0.05
$\frac{\text{Liver weight}}{\text{Body weight}} \times 100$	12	3.85 ± 0.12 (15)	4.68 ± 0.15 (15)	<0.01
	18	3.59 ± 0.18 (4)	4.42 ± 0.21 (4)	<0.05
Protein concentration[a], mg./g. liver ± *SE*	12	22.97 ± 1.03 (6)	16.78 ± 0.79 (6)	<0.01
	18	29.25 ± 2.28 (4)	15.53 ± 0.33 (4)	<0.01

[a] 105,000×*g* microsomal pellet. Number in parentheses is number of animals in group.

Table II—Effect of Ascorbic Acid Deficiency on Activities of Selected Hepatic Enzymes

Enzyme and Substrate	Diet[a]	Substrate Metabolized, μmoles/g. Liver/hr. ± SE
Aniline hydroxylase[b] (aniline)	Control	1.645 ± 0.219
	Ascorbic acid deficient	0.871 ± 0.077[c]
Hexobarbital oxidase[b] (hexobarbital)	Control	6.629 ± 0.987
	Ascorbic acid deficient	3.888 ± 0.196[c]
Glucose-6-phosphate dehydrogenase[d] (NADP)	Control	393.6 ± 38.4
	Ascorbic acid deficient	377.5 ± 50.1

[a] Animals on diet 12 days. [b] 9000×g supernate. [c] Significantly different from control ($p < 0.05$). [d] 105,000×g supernate.

indicated that in ascorbic acid deficiency states, drug hydroxylation by liver microsomes is markedly decreased (1–4), whereas nonsignificant decreases in *N*- and *O*-demethylation and nitro and azo reduction have been noted (2). Since previous studies were conducted at fixed single-substrate concentrations, there is little evidence to indicate whether these effects result from decreased affinity or decreased enzyme concentration. Although there appears to be no reduction in NADPH–cytochrome c reductase or NADH–cytochrome c reductase (2), there is a discrepancy as to whether cytochromes P-450 and b_5 are altered (2, 3). This study will focus on the effects of ascorbic acid (vitamin C) deficiency on the kinetics of drug hydroxylation, the inductive effects of sodium phenobarbital on these kinetics, the rate of recovery of drug hydroxylation activity following injections of ascorbic acid, and the effects of ascorbic acid deficiency on some electron transport components.

MATERIALS AND METHODS

Animals and Diet—Male albino guinea pigs (280 ± 46 g.) were fed a pelleted ascorbic acid-deficient diet[1] for 12 or 18 days before sacrifice. The diet consisted of: ground oats, 40.0%; ground bran, 15.0%; alfalfa, 8.0%; sodium chloride, 0.5%; calcium carbonate, 1.0%; and magnesium sulfate, 0.5%. The control animals received daily injections of ascorbic acid (20 mg./kg. i.p.), and the deficient ones received sterile normal saline (1 ml./kg.). Microsomal enzyme induction was accomplished by the injection of sodium phenobarbital (50 mg./kg. i.p.) on the 8th through 11th days of the dietary treatment. Unless otherwise stated, all animals were killed 24-hr. after the last injection.

Preparation of Hepatic Microsomes—The animals were stunned by a sharp blow to the neck and decapitated; the livers were excised, washed in cold 1.15% potassium chloride solution, blotted, weighed, and put into three volumes of cold 1.15% potassium chloride solution packed in ice. Then the livers were cut into small pieces and homogenized with a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at 9000×g for 20 min. in a refrigerated centrifuge[2] to remove nuclei, mitochondria, and unbroken cells. Washed microsomes were prepared by centrifuging this supernate at 105,000×g for 60 min., resuspending in 1.15% potassium chloride, and recentrifuging.

Assay of Drug-Metabolizing Activities—Unless otherwise stated, incubation mixtures contained 5 mg. microsomal protein, 2.0 μmoles NADP, 25.0 μmoles glucose-6-phosphate, 25 μmoles magnesium sulfate, 2 units glucose-6-phosphate dehydrogenase, and sufficient 0.1 *M* phosphate buffer (pH 7.4) to make 5.0 ml. When 9000×g supernates were used, the glucose-6-phosphate dehydrogenase was omitted from the incubation mixture and the enzyme was equivalent to 250 mg. liver. As drug substrates, the incubation mixtures contained 4.0, 1.0, 0.4, or 0.2 μmoles of hexobarbital; 2.5, 1.0, 0.5, or 0.25 μmoles of aniline; 4.0, 2.0, 1.0, or 0.5 μmoles of zoxazolamine; or 50.0, 5.0, 1.0, or 0.5 μmoles of ethylmorphine. All substrates were incubated at 37° for 20 min. under air in a metabolic incubator[3] at 125 oscillations/min.

The aliphatic hydroxylation of hexobarbital was determined by measuring the disappearance of substrate according to the method of Cooper and Brodie (5). Aromatic hydroxylation of aniline was determined by measuring the formation of *p*-aminophenol, using a modification of the method of Kato and Gillette (6). Aromatic hydroxylation of zoxazolamine was determined by measuring the disappearance of substrate, using the method of Conney *et al.* (7) and Jauchau *et al.* (8). *N*-Demethylation of ethylmorphine was determined by measuring the formation of formaldehyde by the procedure of Nash (9). The NADPH–cytochrome c reductase activity of hepatic microsomes was determined by the method of Phillips and Langdon (10).

Liver microsomal protein was determined by the biuret procedure of Gornall *et al.* (11). Glucose-6-phosphate dehydrogenase activity of the 105,000×g supernate was measured by the method of Glock and MacLean (12).

Estimation of Microsomal Cytochromes P-450 and b_5 and Their Reactivity—The cytochrome P-450 and b_5 contents of hepatic microsomes (resuspended in 1.15% potassium chloride containing 0.05 *M* tromethamine buffer at pH 7.4 and at a constant protein concentration of 2.5 mg./ml.) was determined by the method of Omura and Sato (13). Ethyl isocyanide difference spectra of reduced hemoprotein P-450 were measured by the method of Sladek and Mannering (14). Spectral shift studies, using aniline and hexobarbital as substrates for estimating binding affinities (K_s) and the maximum spectral change ($A_{max.}$) were performed by the method of Remmer *et al.* (15), using microsomes diluted to 2 mg. protein/ml. with 0.3 *M* phosphate buffer at pH 7.4. These measurements were made with a dual wave length-splitbeam spectrophotometer[4] using the appropriate mode.

Statistics—The maximum rate of metabolism ($V_{max.}$) and K_m for aniline hydroxylase, hexobarbital oxidase, and ethylmorphine demethylase were calculated using the computer program of Wilkinson (16), as were the binding constant K_s and its $A_{max.}$ for hexobarbital and aniline.

RESULTS AND DISCUSSION

Body and Liver Weights and Microsomal Protein Concentrations—After 12 and 18 days on the deficient diet, there were significant reductions in terminal body weight and significant increases in liver weight–body weight ratios in the ascorbic acid-deficient animals. The microsomal protein (105,000×g pellet) per gram liver in deficient guinea pigs was 75% that of controls at 12 days and 53% that of controls at 18 days on the diet (Table I).

Glucose-6-phosphate dehydrogenase activity of the 105,000×g supernatant fraction was not altered by the deficiency state (Table II).

The hydroxylations of hexobarbital and aniline at one substrate concentration by 9000×g supernatant fractions were significantly less in the ascorbic acid-deficient guinea pigs (Table II). This finding verified the results obtained by Kato *et al.* (2). The apparent K_m and $V_{max.}$ for aniline hydroxylase, hexobarbital oxidase, and ethylmorphine demethylase are shown in Table III. In ascorbic acid-deficient animals, the apparent $V_{max.}$ for aniline hydroxylation was significantly depressed when calculated as micromoles per gram liver and when measured as metabolism per unit of microsomal protein. This reduction in the rate of aniline hydroxylation is due to a decrease in concentration of microsomal protein and P-450 brought on by the deficiency. Since the apparent K_m for aniline hydroxylation was not changed, it is doubtful that this treatment produced qualitative changes in the composition of the enzyme or

[1] Nutritional Biochemical Corp.
[2] Sorvall RC2-B.
[3] Dubnoff.
[4] Aminco-Chance.

Table III—Effect of Ascorbic Acid Deficiency on the Apparent $V_{max.}$ and K_m of Aniline, Hexobarbital, and Ethylmorphine Metabolism

Substrate	Diet[a]	$V_{max.}$[b], μmoles Metabolized/g. Liver/hr. ± *SE*	K_m, m*M* ± *SE*	$V_{max.}$[c], nmoles Metabolized/mg. Protein/hr. ± *SE*	K_m, m*M* ± *SE*
Aniline	Control	1.90 ± 0.21	0.68 ± 0.18	174.61 ± 30.09	0.56 ± 0.14
	Ascorbic acid deficient	0.99 ± 0.12[d]	0.61 ± 0.17	86.20 ± 14.83[d]	0.49 ± 0.13
Ethylmorphine	Control	—	—	300.66 ± 29.05	0.36 ± 0.12
	Ascorbic acid deficient	—	—	247.70 ± 29.85	0.29 ± 0.12
Hexobarbital	Control	—	—	878.52 ± 395.58	2.95 ± 1.29
	Ascorbic acid deficient	—	—	205.42 ± 40.78	0.60 ± 0.16

[a] Animals on diet 18 days. [b] 9000×*g* supernate. [c] 105,000×*g* washed microsomes. [d] Significantly different from control ($p < 0.05$).

Table IV—Effect of Ascorbic Acid Deficiency on Aniline and Hexobarbital Binding to Washed Microsomes

Diet	Aniline		Hexobarbital	
	K_s (m*M*)	$A_{max.}$ (Δ*A*/mg. Protein)	K_s (m*M*)	$A_{max.}$ (Δ*A*/mg. Protein)
Control	0.325 ± 0.045	0.022 ± 0.001	0.386 ± 0.030	0.050 ± 0.002
Ascorbic acid deficiency	0.315 ± 0.045	0.021 ± 0.001	0.412 ± 0.049	0.034 ± 0.002[a]

[a] Significantly different from control ($p < 0.05$).

Table V—Effect of Ascorbic Acid Deficiency on Content of Microsomal Cytochromes P-450 and b_5 and on Ethyl Isocyanide Binding Spectra

	Diet	
	Control	Ascorbic Acid Deficient
Cytochrome P-450 content (nmoles/mg. protein ± *SE*)	1.676 ± 0.027	1.311 ± 0.079[a]
Cytochrome b_5 (nmoles/mg. protein ± *SE*)	0.984 ± 0.051	1.002 ± 0.063
Ethyl isocyanide spectra (Δ*A*/mg. protein/ml.)		
Δ430–490 nm.	0.100 ± 0.003	0.122 ± 0.013
Δ455–490 nm.	0.076 ± 0.001	0.087 ± 0.013
Ratio: 430/455	1.32 ± 0.03	1.43 ± 0.06

[a] Significantly different from control ($p < 0.05$).

in its affinity for, or reactivity with, the substrate. This was verified by the apparent dissociation constants (K_s) and maximal spectral shifts ($A_{max.}$) for aniline (Table IV).

The $V_{max.}$ or K_m for ethylmorphine demethylase was not altered in ascorbic acid deficiency; however, hexobarbital oxidase activity per unit of protein appeared to be reduced at the four substrate concentrations used. This, coupled with the fact that the maximal spectral shift ($A_{max.}$) for hexobarbital was significantly depressed (Table IV) in the ascorbic acid-deficient animals, indicates that a qualitative change is produced in this metabolic system. Thus, it appears that oxidative enzymes of hepatic microsomes responsible for aniline and ethylmorphine metabolism depend upon ascorbic acid for their synthesis or stability, whereas hexobarbital oxidase may also be altered in its reactivity with its substrate.

The cytochrome P-450 content per unit of microsomal protein is significantly reduced in the ascorbic acid-deficient guinea pig (Table V). The use of ethyl isocyanide to differentiate cytochrome P-450 from P_1-450 has been proposed (14). The data obtained using this ligand indicate that the two forms of cytochrome P-450 are equally sensitive to ascorbic acid deficiency (Table V). Thus, although total cytochrome P-450 is depressed, it is doubtful that its qualitative properties are altered. From these studies it appears that the limiting factor in ethylmorphine metabolism is the quantity of microsomal enzyme, but the reduction in metabolism of hexobarbital and aniline may be dependent upon both protein and cytochrome P-450 loss in the ascorbic acid-deficient pigs.

Effects of Ascorbic Acid Injections at Timed Intervals on Recovery from An Ascorbic Acid-Deficiency State—Twenty-five guinea pigs were used for this study, all maintained on an ascorbic acid-deficient diet as in the previous experiment. Nine control and 16 deficient animals were injected as described previously.

After 12 days the deficient animals were injected with ascorbic acid (20 mg./kg. i.p.) at 24, 16, 8, 4, and 1 hr. before decapitation. Two animals received no ascorbic acid.

Differences in liver weight–body weight ratios between deficient and control animals were not resolved within the 24-hr. period following one injection of ascorbic acid (Table VI). Although the results were not entirely consistent, the cytochrome b_5 content ap-

Table VI—Recovery of Selected Hepatic Components and Functions following Ascorbic Acid Injections in Deficient Animals

Component Analyzed (Mean ± *SE*)	Deficient	Time of Ascorbic Acid Injection Prior to Decapitation					Controls
		1 hr.	4 hr.	8 hr.	16 hr.	24 hr.	
Protein[a], mg./g. liver	23.0 ± 1.0	21.8 ± 3.0	39.4 ± 5.4	16.0	16.0	24.0	29.0 ± 1.3
Liver weight / Body weight × 100	5.20 ± 0.72[b]	4.61 ± 0.11[b]	4.83 ± 0.32[b]	5.44 ± 0.99[b]	5.18 ± 0.33[c]	5.07 ± 0.35[b]	4.06 ± 0.12
Cytochrome P-450[d]	0.46 ± 0.05[c]	0.54 ± 0.05[b]	0.77 ± 0.02	—	—	0.75	1.01 ± 0.06
Cytochrome b_5[d]	0.52 ± 0.03[b]	0.62 ± 0.06	0.76 ± 0.01	—	—	0.70	0.72 ± 0.03
NADPH–cytochrome c reductase[e]	25.18 ± 6.58	18.61 ± 0.64	17.49 ± 1.13	—	—	27.5	22.59 ± 2.28

[a] 105,000×*g* microsomal pellet, mg./g. liver. [b] Significantly different from control ($p < 0.05$). [c] Significantly different from control ($p < 0.01$). [d] nmoles/mg. microsomal protein. [e] nmoles/mg. protein/min.

Table VII—Activities of Selected Hepatic Enzymes following Ascorbic Acid Injections in Deficient Animals

Enzyme Analyzed (Mean ± SE)[a]	Deficient	Time of Ascorbic Acid Injection Prior to Decapitation: 1 hr.	4 hr.	8 hr.	16 hr.	24 hr.	Controls
Aniline hydroxylase	1.22 ± 0.19[b]	1.70 ± 0.30	1.74 ± 0.41	1.28 ± 0.12[b]	1.56 ± 0.08[c]	1.79 ± 0.03	2.34 ± 0.12
Ethylmorphine demethylase	—	1.77 ± 0.35	0.96 ± 0.32[c]	—	0.79	1.58	2.53 ± 0.19
Hexobarbital oxidase	1.35 ± 0.26[b]	2.95 ± 0.41	3.36 ± 0.04	1.87	1.76	2.86	3.67 ± 0.15
Zoxazolamine oxidase	—	1.88 ± 0.99	—	0.57	—	0.25	2.23 ± 0.90

[a] μmoles substrate metabolized/g. liver/hr. [b] Significantly different from control ($p < 0.01$). [c] Significantly different from control ($p < 0.05$).

peared to be restored 4 hr. following injection; however, cytochrome P-450 appeared to be depressed for up to 24 hr. NADPH–cytochrome c reductase was not altered by the deficiency state.

The altered drug metabolism may require more than a single injection of ascorbic acid to reverse the depression induced by 12 days of the ascorbic acid-deficient diet (Table VII). When comparing $V_{max.}$ data, it was found that the maximum rate of aniline metabolism per gram liver was not reversed by one injection of ascorbic acid given at 1, 4, 8, 16, or 24 hr. prior to decapitation. The $V_{max.}$ for ethylmorphine demethylase was consistently about 57% that of corresponding control animals, whereas the $V_{max.}$ for zoxazolamine hydroxylase was not different as a result of the deficiency state. The K_m for the reaction was altered only with respect to aniline, where it was significantly less than controls at the 4- and 16-hr. injection periods.

Inductive Effects of Sodium Phenobarbital on Kinetics of Hepatic Microsomal Drug Hydroxylation—Twelve male albino guinea pigs were fed the ascorbic acid-free diet for 12 days. Six of these were injected with ascorbic acid as described previously. On each of the last 4 days of the diet, all animals were injected with sodium phenobarbital (50 mg./kg. i.p.). The animals were killed 24 hr. after the last injection (12th day), and the liver microsomes were prepared and analyzed. The activities of microsomes from deficient guinea pigs were induced to the same level as the controls (Table VIII). Thus, ascorbic acid deficiency does not inhibit induction of aniline hydroxylase by phenobarbital.

Although the apparent $V_{max.}$ and K_m for ethylmorphine demethylase were approximately twice as great in the ascorbic acid.

Table VIII—Effects of Ascorbic Acid Deficiency on Kinetics of Microsomal Drug Hydroxylation in Sodium Phenobarbital[a]-Treated Animals

Substrate	Diet[b]	$V_{max.}$, μmoles Metabolized/g. Liver/hr. ± SE	K_m, mM ± SE
Aniline	Control	10.39 ± 0.94	3.54 ± 0.59
	Ascorbic acid deficient	10.80 ± 0.98	3.54 ± 0.98
Ethylmorphine	Control	21.5 ± 8.0	47.9 ± 22.1
	Ascorbic acid deficient	11.8 ± 3.6	22.6 ± 10.6

[a] Sodium phenobarbital (50 mg./kg./day i.p.) for 4 days prior to decapitation. [b] Animals on diet for 12 days.

treated as in the deficient group, these differences were not statistically significant. Liver weight–body weight ratios in the two groups were not significantly different. These results are in agreement with those of Leber *et al.* (3), who found that phenobarbital induction of aminopyrine demethylase was at least equal in scorbutic animals as in controls whereas greater induction of cytochrome P-450 occurred in scorbutic animals than in controls.

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NUTRI. L VALUE OF CANNED FOODS. VII. EFFECT OF COMMERCIAL CANNING AND SHORT-TIME STORAGE ON ASCORBIC ACID CONTENT OF GRAPEFRUIT JUICE[1]

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From a nutritional viewpoint the value of canned grapefruit juice is largely related to ascorbic acid content. Consequently the preservation of this factor is of the utmost importance to both the canner and the consumer of this product. Floyd and Fraps (1942) have reported a group of studies on the ascorbic acid content of fresh and canned grapefruit juices from the Rio Grande Valley of Texas. These investigators compared the average ascorbic acid content of 109 samples of canned grapefruit juice with the average content of 21 samples of grapefruit juice expressed from orchard fruit. While the raw and canned juices were not operationally related, the authors concluded that the average loss of ascorbic acid in commercial grapefruit-juice canning operations was 18.2 per cent.

More recently in an extensive survey of Florida grapefruit juice canneries, Moore, Wiederhold, Atkins, and MacDowell (1944) found that the average loss of ascorbic acid in the manufacture of canned grapefruit juice was only three per cent. In these experiments juice sampled at several points in the canning line was analyzed, the first sample being taken from the freshly extracted juice and the last from the canned product. By proper adjustment of sampling times, they were able to sample a given lot of juice wherever desired without interrupting the rate of flow through the cannery. As the result of a similar type of study, Ross (1944) has reported that freshly canned grapefruit juice retains 92 to 98 per cent of the original ascorbic acid content.

A detailed survey of the ascorbic acid retention in grapefruit juice during its preparation and canning was conducted in the Rio Grande Valley to obtain more information on this subject.

EXPERIMENTAL PROCEDURE

Through the co-operation of the Texas Canners' Association arrangements were made with the operators of canneries to carry out these studies in 12 plants. The operations during one day were surveyed at each of 10 plants. At the other two plants (VI and XI) the study was continued for an additional two days in order to check on day-to-day variations.

Information on the source and history of the particular fruit canned, the equipment used, and the canning operations was obtained at each plant.

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The time required for each step in the canning operation was determined to insure representative sampling in each lot of juice. Juice from selected lots of fruit was then sampled at predetermined points in the canning line.

Samples were taken consecutively (1) from the freshly extracted juice, (2) after screening, (3) at the holding tank outlet whenever possible, (4) from the pasteurized juice at the filler bowl, and (5) from the cooled cans. In some instances the arrangement of the canning line did not permit taking a sample after holding before the juice entered the pasteurizers. Additional sampling stations were necessary at Plant I which used deaeration and at Plant III which used a small heated holding tank for pasteurized juice (Table 1).

Both "step-by-step" runs and "over-all" runs were made. In the step-by-step runs samples were taken at every sampling station. In the first part of the survey over-all runs were made by sampling the freshly extracted juice and the final canned product. Later, for reasons explained under sampling procedure, the first sample for over-all runs was taken after screening.

Two sampling procedures were used. In Procedure A samples were prepared by measuring 10-ml. portions of stabilizing solution (three per cent metaphosphoric acid and eight per cent acetic acid in copper-free water) into 50-ml. Erlenmeyer flasks. Ten ml. of collected juice was then pipetted into each flask. Aliquots for titration were drawn from these preparations. Four or five such samples were taken at each station over a 10-minute period. For sampling Procedure B, 50 ml. of stabilizing solution were measured into a 100-ml. volumetric flask. Samples of approximately five ml. were taken once a minute from the line for 10 minutes so that the flask was filled to the mark. Aliquots were taken from the mixed contents of the flask for titration. This procedure permitted a larger number of samplings in each run, is simpler, and seems less subject to error than Method A. For analysis of the canned product six of the cooled cans were collected from each lot of juice over a 10-minute period. These were analyzed on the same day as packed. The cans were shaken thoroughly, opened, and sampled immediately. Under sampling Procedure A, a separate sample was prepared from each can. Under Procedure B, aliquots of approximately eight and one-third ml. were pipetted from each can into the sampling flask so as to fill it to the mark.

The freshly extracted juice was prepared for the above sampling procedures by filtering through coarse gauze. It was noticed after a few runs that the screened juice tended to have a higher ascorbic acid content than the freshly extracted juice prepared in this manner. This seems to be due to the fact that the screened juice contains more pulp than the filtered samples. Since this pulp or "rag" has a higher ascorbic acid content than the juice alone, the ascorbic acid content of the screened juice will vary with its pulp content. For this reason it was felt that the screened juice was more representative of the canned product than the freshly extracted juice. Therefore, the later over-all runs were made by sampling the screened juice and the canned juice. The per cent retention of ascorbic acid during canning has been calculated on the basis of the content of the

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screened sample as much as possible. Retention of ascorbic acid was calculated on the basis of the freshly extracted juice wherever the screened juice was not sampled. For the sake of uniformity, the latter figures (in parentheses in Table 1) have been excluded from the averages. All samples have been analyzed by titration with 0.02 per cent aqueous sodium-2,6-dichlorobenzenoneindophenol solution which was standardized daily against crystalline ascorbic acid (Merck).

In addition to the six cans of juice that were analyzed immediately after canning, six more were collected from each step-by-step run and stored for 40 days at room temperature of 18.3 to 26.7°C.(65 to 80°F.). At the end of the storage period measurements of headspace, vacuum, Brix, and titratable acidity were made. Aliquots were taken according to Procedure B and analyzed for ascorbic acid.

RESULTS AND DISCUSSION

The average retention of ascorbic acid during canning operations for all plants was 96.5 per cent (Table 1). This value checks very well with the figure (97 per cent) reported by Moore *et al.* (1944). Average retention for individual plants ranged from 92.2 per cent for Plant III to 99.8 per cent for VI (first day's operations). The daily average retentions for Plants VI and XI are quite uniform (98.7, 97.8, and 96.9 per cent for Plant VI; 97.2, 96.0, and 97.1 per cent for Plant XI). The low retention in Plant III appears to be due to the practice of holding the hot juice after pasteurization. The line arrangement of this plant did not permit the taking of samples immediately before pasteurizing, but the small losses incurred in holding tanks before pasteurization at other plants indicate that the loss occurs in the heated juice. With the exception of this case, the magnitude of the losses of ascorbic acid during canning did not seem great enough to justify any attempt to correlate them with differences in the canning equipment.

The retention of ascorbic acid during the storage period was calculated on the basis of the amounts found immediately after canning and at the end of the storage period. Individual samples retained from 83 to 100 per cent of their ascorbic acid. The average retention for all stored samples was 91.3 per cent. The differences in retention could not be definitely correlated with Brix, titratable acidity, headspace, or vacuum. The upper part of the range compares with values reported by Ross (1944) on the retention of ascorbic acid in canned, unsweetened grapefruit juice after one month's storage at room temperature. More complete work on the effect of storage at various temperatures on the ascorbic acid content of grapefruit juice is being carried out elsewhere in the nutrition program of which this study is a part.

SUMMARY

The effect of commercial canning practice on the ascorbic acid content of grapefruit juice has been studied in 12 canneries of the Rio Grande Valley. The retention of ascorbic acid during canning ranged from 92.2 to 99.6 per cent with an average retention for all plants of 96.7 per cent. During subsequent storage for 40 days (average about 72°F.) samples of

TABLE 1

Ascorbic Acid Content and Retention During Canning and After 40-Day Storage of Grapefruit Juice From Rio Grande Valley Canning Plants

Plant and runs	Freshly extracted	After screening	From holding tank	From filler bowl	Canned juice immediately after cooling		Sampling method	Stored 40 days	
					Ascorbic acid	Retained		Ascorbic acid	Retained[1]
				DEAERATOR					
	mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*pct.*		*mg./100 ml.*	*pct.*
I—1	39.7	40.0	40.6	38.9	41.4	40.8	102.0	A	38.4	94.0
2	44.7	42.9	42.9	41.3	43.4	41.5	96.8		40.6	98.0
Av.	42.2	41.4	41.7	40.1	42.4	41.1	99.4		39.5	96.0
II—1	47.1	51.0	49.1	.....	49.5	53.0	103.9	A	47.5	89.5
2	(48.0)	.....	.....	.....	.....	(50.4)	(105.0)		.....	.....
3	50.4	51.6	51.3	.....	45.0	46.4	90.0		44.9	96.8
Av.	48.7	51.3	50.5	.....	47.2	49.7	96.9		46.2	93.1
				PASTEURIZED[2]					
III—1	43.7	46.0	.....	40.7	41.0	42.3	91.9	A	.....	.....
2	41.3	42.8	.....	41.0	39.2	39.4	92.1		37.2	94.5
3	43.5	45.5	.....	43.2	43.1	42.4	92.7		38.9	91.8
Av.	42.8	44.8	.....	41.6	41.1	41.3	92.2		38.0	93.1
IV—1	42.9	43.2	43.2	.....	40.4	39.8	92.1	A	36.8	92.5
2	(42.6)	.....	.....	.....	.....	(41.3)	(96.8)		.....	.....
3	(40.0)	.....	.....	.....	.....	(40.3)	(99.3)		.....	.....
4	42.4	43.1	41.7	.....	42.5	41.1	95.4		39.8	97.0
Av.	42.6	43.1	42.4	.....	41.4	40.4	93.7		38.3	94.7
V—1	38.5	39.4	39.3	.....	36.5	36.9	92.6	A	35.7	96.8
2	(35.9)	.....	.....	.....	.....	(35.7)	(95.5)		.....	.....
3	36.9	38.0	38.2	.....	36.5	36.5	96.1		35.6	97.6
Av.	37.7	38.7	38.7	.....	36.5	36.7	94.3		35.6	97.2
VI—1	37.9	37.6	.....	.....	37.7	37.5	99.7	A	37.2	99.3
2	(37.6)	.....	.....	.....	.....	(37.5)	(99.7)		.....	.....
(a) 3	(38.7)	.....	.....	.....	.....	(37.0)	(95.4)		.....	.....
4	36.4	36.5	.....	.....	36.5	36.4	100.0		36.4	100.0
Av.	37.1	37.0	.....	.....	37.1	36.9	99.8		36.8	99.6

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TABLE 1 *(Concluded)*

Plant and runs	Freshly extracted	After screening	From holding tank		From filler bowl	Canned juice immediately after cooling		Sampling method	Stored 40 days	
						Ascorbic acid	Retained		Ascorbic acid	Retained[1]
	mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*pct.*		*mg./100 ml.*	*pct.*
XI—1	.....	40.0	.....	.....	.....	38.7	96.7	B	.....	.....
2	.....	40.7	.....	.....	.....	36.0	88.5		.....	.....
(a) 3	38.5	38.0	.....	.....	38.0	38.0	100.0		35.3	93.0
4	.....	39.0	.....	.....	.....	38.0	97.5		.....	.....
5	39.4	39.7	.....	.....	38.6	41.1	103.5		33.7	83.0
Av.	38.9	39.5	.....	.....	38.3	39.4	97.2		34.5	88.0
XI—1	40.2	40.9	.....	.....	39.6	39.0	95.3	B	35.5	89.7
2	.....	40.8	.....	.....	.....	39.1	95.5		.....	.....
(b) 3	.....	39.4	.....	.....	.....	37.8	96.0		.....	.....
4	.....	39.4	.....	.....	.....	38.4	97.5		.....	.....
5	39.5	39.8	.....	.....	39.3	38.0	95.5		34.7	94.0
Av.	39.8	40.1	.....	.....	39.4	38.5	96.0		35.1	91.8
XI—1	40.4	40.6	.....	.....	39.2	38.4	94.7	B	33.1	86.3
2	.....	40.5	.....	.....	.....	40.2	99.5		.....	.....
(c) 3	40.0	40.6	.....	.....	38.8	38.4	94.8		33.6	87.6
4	.....	40.3	.....	.....	.....	39.0	97.0		.....	.....
5	.....	39.0	.....	.....	.....	38.8	99.3		.....	.....
Av.	40.2	40.2	.....	.....	39.0	39.0	97.1		33.3	86.9
XII—1	.....	47.1	.....	.....	.....	44.7	95.0	B	.....	.....
2	42.2	43.1	42.8	.....	41.0	40.9	95.2		34.3	83.8
3	.....	41.7	.....	.....	.....	40.0	96.0		.....	.....
4	39.4	40.6	40.6	.....	39.4	39.2	96.7		35.0	89.5
Av.	40.8	43.1	41.7	.....	40.2	41.2	95.7		34.6	86.6
Average of all juices sampled[3]	40.7	40.9	.....	.....	39.7	39.4	96.5		36.0	91.3

NOTE: The juice going through the screening operation in Plant III represents a combination of the first extracted juice and a second extraction made on the recovered pulp with a screw extractor. Ascorbic acid content of second extract: mg./100 ml. = 47.5, 51.1, 51.0 (Av. 49.9).

[1] The per cent retentions during storage were calculated on the basis of the amounts in the canned juices immediately after canning and at the end of the storage period. [2] Held in heated tank after pasteurization. [3] Excepting individual values in parentheses.

VI—1	38.6	38.4	.....	.....	37.6	37.7	98.0	B	32.1	85.2
2	40.3	38.4	.....	.....	39.7	37.3	96.3		33.6	90.0
(b) 3	.....	37.7	.....	.....	.....	36.5	96.9		.....	.....
4	.....	41.3	.....	.....	.....	40.3	97.6		.....	.....
5	.....	40.5	.....	.....	.....	39.0	96.3		.....	.....
Av.	38.2	38.7	.....	.....	37.9	37.7	97.8		32.8	87.6
VI—1	.....	39.3	.....	.....	.....	36.2	92.1	B	.....	.....
2	.....	37.1	.....	.....	.....	37.4	100.7		.....	.....
(c) 3	.....	40.2	.....	.....	.....	38.5	95.8		.....	.....
4	41.2	41.4	.....	.....	40.8	40.6	98.1		34.7	85.3
5	39.2	39.6	.....	.....	38.7	38.6	97.6		37.2	96.5
Av.	40.2	39.5	.....	.....	39.1	38.2	96.9		35.9	90.9
VII—1	40.1	39.9	41.1	.....	39.8	39.8	99.2	B	35.2	88.5
2	(42.5)	.....	.....	.....	.....	(41.7)	(98.1)		.....	.....
3	(41.0)	.....	.....	.....	.....	(39.4)	(96.0)		.....	.....
4	(40.5)	.....	.....	.....	.....	(38.8)	(95.8)		.....	.....
5	39.4	39.1	38.3	.....	37.0	36.9	94.2		32.3	87.6
Av.	39.7	39.5	39.7	.....	38.4	38.3	96.7		33.7	88.0
VIII—1	39.3	39.6	39.4	.....	37.8	37.3	94.2	B	33.3	89.3
2	39.3	40.4	40.7	.....	39.0	38.9	96.3		34.3	88.2
3	40.0	41.7	42.9	.....	40.1	40.3	96.6		.....	.....
4	.....	43.1	.....	.....	.....	41.6	96.5		.....	.....
5	.....	43.0	.....	.....	.....	41.5	96.5		.....	.....
Av.	39.5	41.5	41.0	.....	39.0	40.1	96.0		33.8	88.7
IX—1	38.0	37.8	.....	.....	36.2	36.4	96.3	B	32.9	90.5
2	37.3	38.4	.....	.....	37.9	37.7	98.2		33.3	88.4
3	.....	40.7	.....	.....	.....	40.4	99.2		.....	.....
4	.....	37.7	.....	.....	.....	36.2	97.6		.....	.....
5	.....	38.1	.....	.....	.....	37.4	98.2		.....	.....
Av.	37.6	38.4	.....	.....	37.0	37.6	97.9		33.1	89.4
X—1	42.7	43.6	42.2	.....	41.5	40.5	92.9	B	36.4	89.8
2	43.0	42.4	40.8	.....	39.6	40.5	95.1		35.8	88.5
3	40.6	40.8	40.5	.....	39.3	39.1	95.8		.....	.....
4	.....	41.4	.....	.....	.....	40.5	97.8		.....	.....
5	.....	40.5	.....	.....	.....	40.4	99.7		.....	.....
Av.	42.1	41.8	41.2	.....	40.1	40.2	96.2		36.1	89.1

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grapefruit juice retained, on the average, 91.3 per cent of the ascorbic acid present in the freshly canned juice.

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Ascorbic Acid Deficiency and Induction of Hepatic Microsomal Hydroxylative Enzymes by Organochlorine Pesticides[1]

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Ascorbic Acid Deficiency and Induction of Hepatic Microsomal Hydroxylative Enzymes by Organochlorine Pesticides. WAGSTAFF, D. J., and STREET, J. C. (1971). *Toxicol. Appl. Pharmacol.* **19**, 10–19. Hepatic microsomal hydroxylative enzyme systems, *O*-demethylation of *p*-nitroanisole and hydroxylation of aniline, were induced by dietary dieldrin and to a lesser extent by DDT and lindane in guinea pigs. However, liver weights were not consistently increased nor were microsomal protein concentrations elevated.

Ascorbic acid deficiency impaired the induction by dieldrin as early as 2 days on deficient diet. As scurvy developed through stages of normal weight gains, depressed gains and finally hemorrhagic lesions, the impairment became consistently lower during the period of depressed weight gains due to the accompanying decline in basal enzyme activity. Sex had no effect on either induction by dieldrin or its impairment in ascorbic acid deficiency. Maintenance of induction was related to dietary levels rather than hepatic concentrations of the vitamin and required higher dietary levels of ascorbic acid than scurvy prevention.

Induction of hepatic microsomal hydroxylative enzyme activity by drugs, pesticides, and other foreign chemicals is recognized as an important adaptative process in animals, a process markedly influencing the toxicity of such agents. The normal activity of hepatic microsomal enzymes is depressed in guinea pigs fed ascorbic acid-deficient diets. Axelrod *et al.* (1954) found increased half-life values in the blood stream for acetanilide, aniline, and antipyrine injected into guinea pigs which had been fed ascorbic acid-deficient diet for 16–20 days. Decreased hydroxylation of acetanilide and coumarin by microsomal preparations from scorbutic guinea pigs were observed by Degkwitz and Staudinger (1965) and Degkwitz *et al.* (1968), respectively. Recently Kato *et al.* (1969) noted that hydroxylation of aniline, hexobarbital, and zoxazolamine were reduced in guinea pigs fed ascorbic acid-deficient diet for 12 days.

The present report describes the interaction of the detrimental effect of ascorbic acid deficient diet and the inductive effect of organochlorine pesticides on hepatic

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icrosomal enzyme activity. Concurrent environmental exposure to pesticides and etary inadequacy of ascorbic acid constitutes a potential public health problem due to npairment of detoxication processes.

METHODS

The basic experimental design was a 2 × 2 factorial arrangement in which the effects of 0 and 2000 ppm ascorbic acid (control diet) and 0 and 25 ppm organochlorine pesticide were compared. The pesticides employed were chromatographically pure *p,p'*-DDT, γ-isomer of hexachlorocyclohexane (lindane), and HEOD (dieldrin). Guinea pigs weighing 200–300 g of either Hartley or mongrel strains were individually caged and fed a basal diet deficient in ascorbic acid purchased from Nutritional Biochemicals Corporation.[3] Pesticides dissolved in petroleum ether and crystalline ascorbic acid were added to this diet. The solvent was allowed to evaporate during hand stirring. Mixing was completed in a mechanical mixer. Male animals were used except where otherwise specified. At the end of the feeding periods, which were of variable lengths, the animals were killed with ether. The liver from each animal was excised and weighed, and a portion was homogenized in 4 parts of a solution containing 1.15% KCl and 0.25% nicotinamide. The liver homogenates were centrifuged at 10,000 *g* for 10 min. Supernatant fluid was assayed on the day of the kill for enzyme activity by *O*-demethylation of *p*-nitroanisole (*O*-demethylase) according to the method of Kinoshita *et al.* (1966) and hydroxylation of aniline (aniline oxidase) after the method of Kato and Gillette (1965). Ascorbic acid in liver tissue was determined by the Geshwind *et al.* (1951) modification of the method of Roe and Kuether (1943). Microsomal protein concentration was determined by the method of Lowry *et al.* (1951) in the pellet formed by centrifuging the 10,000 *g* supernatant fluid for 1 hr at 106,000 *g*. Statistical treatment was according to Ostle (1963).

RESULTS

The hydroxylative enzyme inductive abilities of DDT, lindane, and dieldrin when fed 25 ppm of the diet were compared in guinea pigs fed normal and ascorbic acid-deficient diets for 15 days (Table 1). At that dose rate, DDT and lindane produced relatively little enzyme induction. Dieldrin was the most potent inducer and was the only esticide used in the following experiments.

All guinea pigs fed ascorbic acid-deficient ration for 15 days, regardless of concurrent esticide, had hemorrhages which were most obvious in the regions adjacent to the nee and hock joints. These animals were considered scorbutic. In scorbutic animals ot fed pesticide, enzyme activity was reduced compared to nonscorbutic animals; e reduction approached statistical significance. Moreover, lindane and DDT proaced no enzyme induction when added to the diets deficient in the vitamin. Even with ieldrin as the inducer, enzyme activity in animals fed ascorbic acid deficient diet was o greater than that of dieldrin-free controls. Despite the small number of animals in some groups, the depressing effect of ascorbic acid deficiency on induction by dieldrin was significant at the 0.05 level in the factorial analysis. Relative liver weights tended to

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be increased in scorbutic animals, particularly in those treated with pesticide. In normal animals, the pesticides tended to reduce relative liver weight, especially in the case of lindane.

TABLE 1

EFFECTS OF ASCORBIC ACID DEFICIENCY AND DIETARY DDT, LINDANE, AND DIELDRIN ON ACTIVITY OF HEPATIC MICROSOMAL HYDROXYLATIVE ENZYMES IN GUINEA PIGS FED 15 DAYS

Treatment	No. of animals	Liver weight[a]	O-Demethylase[b]	Aniline[c] oxidase
Control diet	5	$5.08^{d,e,f} \pm 0.45$	$140^{d,e} \pm 13$	$35^{d,e,f} \pm 4$
Ascorbic acid-deficient (AAD) diet	4	$5.78^{d,e} \pm 0.36$	$74^{e,f} \pm 4$	$26^{e,f} \pm 3$
DDT (25 ppm) in control diet	5	$4.53^{f,g} \pm 0.34$	$169^{d} \pm 16$	$49^{d,e} \pm 9$
DDT (25 ppm) in AAD diet	3	$6.17^{d} \pm 0.27$	$66^{f} \pm 10$	$23^{f} \pm 2$
Lindane (25 ppm) in control diet	5	$3.89^{g} \pm 0.24$	$185^{d} \pm 16$	$55^{d} \pm 4$
Lindane (25 ppm) in AAD diet	4	$5.33^{d,e,f} \pm 0.29$	$77^{e,f} \pm 6$	$22^{f} \pm 2$
Dieldrin (25 ppm) in control diet	4	$4.87^{e,f,g} \pm 0.33$	$339^{h} \pm 46$	$82^{h} \pm 12$
Dieldrin (25 ppm) in AAD diet	3	$5.93^{d,e} \pm 0.13$	$127^{d,e,f} \pm 37$	$45^{d,e} \pm 15$

[a] Mean ± SE as g liver weight/100 g body weight.
[b] Mean ± SE as μmoles × 10^2 *p*-nitrophenol formed/g liver/hr of incubation.
[c] Mean ± SE as μmoles × 10^2 *p*-aminophenol formed/g liver/30 min of incubation.
[d–h] Means for a particular variable which do not have any superscripts in common are significantly different at the 0.05 level (Modified Duncan Multiple Range Test).

In order to eliminate problems associated with the anorexia and weight loss of frank scurvy, 2 experiments of shorter length were conducted using the basic 2 × 2 factorial arrangement. The experiments ran 12 and 14 days with dieldrin being fed the last 4 days of each experiment. Body weight gains were reduced about one-third in guinea pigs fed ascorbic acid-deficient diets but no other evidence of frank scurvy was seen. Results (Table 2) were similar to the initial experiment. Dieldrin induced both *O*-demethylase and aniline oxidase activities. However, this inductive effect of dieldrin was somewhat suppressed by the ascorbic acid deficiency. For *O*-demethylase the *F*-value of the dieldrin-ascorbic acid deficiency interaction in factorial analysis was significant at the 0.05 level in the 14-day experiment but not significant at the same level in the 12-day experiment. Liver weights were increased by dieldrin in the 14-day experiment but slightly reduced in the 12-day experiment.

Time-course experiments were performed to determine the onset of the impairment of induction by the vitamin deficiency. Animals on the basic 2 × 2 factorial arrangement were killed after 2, 4, and 6 days in one experiment and after 4, 8, and 12 days in a second trial. Dieldrin was fed the entire length of the experimental period for each group. No depression of body weight gains or other evidence of scurvy was seen in these time course experiments. The detrimental effect of the ascorbic acid deficiency on the

duction process in each time period was calculated as the percent impairment of duction. Enzyme activities of animals fed the various diets were used in the following rmula:

$$1 - \left[\frac{\text{Dieldrin (25 ppm) in ascorbic acid-deficient diet} - \text{Control}}{\text{Dieldrin (25 ppm)} - \text{Control}}\right] \times 100 = \text{\% impairment of induction}$$

TABLE 2

EFFECTS OF ASCORBIC ACID DEFICIENCY AND DIELDRIN ON ACTIVITY OF HEPATIC MICROSOMAL HYDROXYLATIVE ENZYMES IN GUINEA PIGS

Treatment	No. of animals	Liver weight[a]	*O*-Demethylase[b]	Aniline oxidase[c]
14-day experiment				
Control Diet	5	4.31 ± 0.28	132 ± 10	29 ± 5
Ascorbic acid-deficient (AAD) diet	5	4.63 ± 0.36	91 ± 10	25 ± 6
Dieldrin (25 ppm) in control diet	5	5.18 ± 0.32	318 ± 22	63 ± 4
Dieldrin (25 ppm) in AAD diet	5	5.43 ± 0.30	172 ± 39	43 ± 7
Factorial analysis	*df*		*F values*	
AAD	1	0.83	16.30[e]	5.09[d]
Dieldrin	1	6.91[d]	33.23[e]	23.42[e]
AAD × dieldrin	1	0.01	5.21[d]	2.00
12-day experiment				
Control Diet	7	4.56 ± 0.22	122 ± 9	31 ± 4
Ascorbic acid-deficient (AAD) diet	9	5.01 ± 0.28	66 ± 6	13 ± 2
Dieldrin (25 ppm) in control diet	7	4.44 ± 0.23	224 ± 20	52 ± 6
Dieldrin (25 ppm) in AAD diet	9	4.84 ± 0.23	141 ± 10	32 ± 3
Factorial analysis	*df*		*F values*	
AAD	1	3.21	36.90[e]	24.38[e]
Dieldrin	1	0.37	58.84[e]	27.76[e]
AAD × dieldrin	1	0.01	1.38	0.64

[a] Mean ± SE as g liver weight/100 g body weight.
[b] Mean ± SE as μmoles × 10^2 *p*-nitrophenol formed/g liver/hr of incubation.
[c] Mean ± SE as μmoles × 10^2 *p*-aminophenol formed/g liver/30 min of incubation.
[d] Significant at 0.05 level.
[e] Significant at 0.01 level.

here was no significant reduction of enzyme activity in animals fed the ascorbic acid-ficient diet containing no dieldrin and thus this group was ignored in the calculation. he values obtained for impairment of *O*-demethylase activity at each time period are otted in Fig. 1. In the lower part of the same figure the liver concentration of ascorbic id is presented for comparison to the values showing impairment of induction. From is figure it is evident that impairment of induction occurred as early as 2 days on the ficient diet. The liver concentration of ascorbic acid, in a corresponding manner, dropped precipitously during the first 2 days but more gradually after that.

Data from these time course studies were analyzed in factorial analysis and the effect of time was removed (Table 3). The negating effect of ascorbic acid-deficiency on induction by dieldrin, expressed in the analysis as the ascorbic acid deficiency-dieldrin

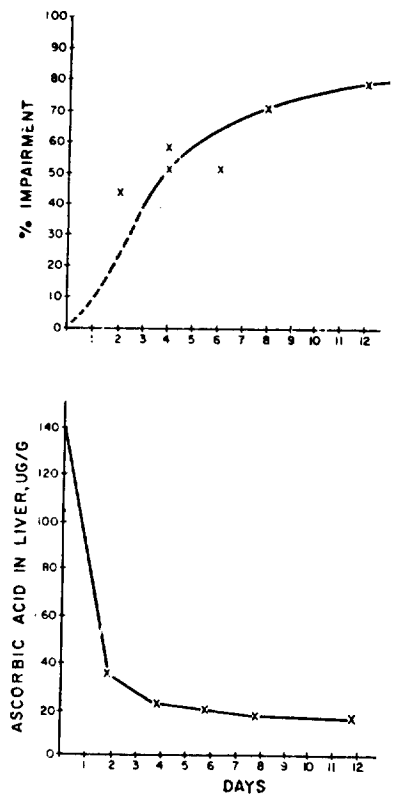

FIG. 1. Impairment of induction of *O*-demethylation of *p*-nitroanisole by dieldrin in guinea pigs fed ascorbic acid-deficient diet for various time periods and concentration of ascorbic acid in liver in these same animals. The points on the impairment curve represent mean values for groups of 5 animals for day 2, 4 (upper point), and 6, and groups of 3 animals for day 4 (lower point), 8, and 12. Impairment was computed as described in the text. Liver ascorbic acid values are the means for the induced animals on the AAD diet.

TABLE 3

F VALUES FROM FACTORIAL ANALYSES OF EFFECTS OF ASCORBIC ACID DEFICIENCY AND DIELDRIN ON ACTIVITY OF HEPATIC MICROSOMAL HYDROXYLATIVE ENZYMES IN GUINEA PIGS FED 2–12 DAYS[a]

Source	Liver weight	*O*-Demethylase	Aniline oxidase
Ascorbic acid deficiency (AAD)	0.64	14.70[b]	13.37[b]
Dieldrin	3.96[c]	71.57[b]	38.89[b]
AAD × dieldrin	0.38	11.13[b]	10.71[b]

[a] Effect of time in factorial analyses of enzymatic activities was not significant (0.05 level) and is not presented.
[b] Significant at 0.01 level.
[c] Significant at 0.05 level.

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interaction, was significant at the 0.01 level for both *O*-demethylase and aniline oxidase. Dieldrin increased liver weight.

There was no effect of sex on either the inductive effect of dieldrin or the depressing effect of ascorbic acid deficiency (Table 4). In factorial analysis, the main effect of sex and all interactions involving sex were not significant at the 0.05 level for either enzyme response.

TABLE 4

EFFECTS OF ASCORBIC ACID DEFICIENCY, DIELDRIN, AND SEX ON ACTIVITY OF HEPATIC MICROSOMAL HYDROXYLATIVE ENZYMES AND HEPATIC MICROSOMAL PROTEIN CONCENTRATION IN GUINEA PIGS FED 12 DAYS

Treatment	No. of animals	Liver weight[a]	*O*-Demethylase[b]	Aniline oxidase[c]	Microsomal protein[d]
Male					
Control Diet	6	4.11 ± 0.27	189 ± 29	36 ± 7	19.8 ± 0.7
Ascorbic acid-deficient (AAD) diet	6	4.07 ± 0.30	142 ± 20	34 ± 4	17.2 ± 1.2
Dieldrin (25 ppm) in control diet	6	4.45 ± 0.14	361 ± 47	78 ± 8	19.3 ± 1.3
Dieldrin (25 ppm) in AAD diet	6	4.39 ± 0.14	313 ± 42	58 ± 6	20.2 ± 0.7
Female					
Control Diet	6	4.47 ± 0.18	197 ± 24	34 ± 4	19.5 ± 1.5
Ascorbic acid-deficient (AAD) diet	6	4.59 ± 0.29	115 ± 13	28 ± 3	17.2 ± 1.4
Dieldrin (25 ppm) in control diet	6	4.35 ± 0.25	416 ± 33	78 ± 8	20.6 ± 0.9
Dieldrin (25 ppm) in AAD diet	6	5.08 ± 0.25	256 ± 33	57 ± 6	19.9 ± 0.9
Factorial analysis	*df*		*F values*		
AAD	1	1.24	14.11[f]	10.77[f]	2.37
Dieldrin	1	2.31	69.91[f]	85.10[f]	4.08
Sex	1	4.64[e]	0.06	0.40	0.05
AAD × dieldrin	1	0.80	0.80	4.97[e]	2.60
AAD × Sex	1	1.96	2.70	0.06	0.22
Dieldrin × sex × AAD	1	0.19	0.03	0.22	0.16

[a] Means ± SE as g liver weight/100 g body weight.
[b] Mean ± SE as μmoles × 10^2 *p*-nitrophenol formed/g liver/hr of incubation.
[c] Mean ± SE as μmoles × 10^2 *p*-aminophenol formed/g liver/30 min of incubation.
[d] Mean ± SE as μg protein/g liver.
[e] Significant at 0.05 level.
[f] Significant at 0.01 level.

In the same experiment, the concentrations of hepatic microsomal protein were slightly depressed in animals fed ascorbic acid-deficient diet. This confirmed observations by Kato *et al.* (1969). Dieldrin added to the control diet had no effect on protein concentration. However, dieldrin in the ascorbic acid-deficient diet seemed to prevent

depression of microsomal protein content. Dieldrin had only a slight effect on liver weight.

The relative effects of ascorbic acid deficiency and dieldrin on *O*-demethylase and their relationship to the development of frank scurvy are summarized in Fig. 2. Ascorbic acid-deficient guinea pigs which were fed dieldrin had lower enzyme activity than those animals fed dieldrin in control diets at every point in time in the various experiments. However, as basal enzyme activity was depressed by ascorbic acid deficiency during the period of lowered body weight gains, the statistical significance of the ascorbic acid deficiency-dieldrin interaction decreased.

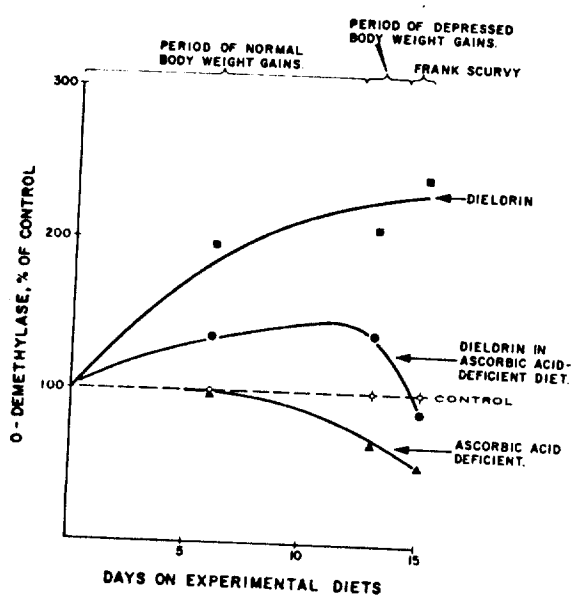

FIG. 2. Idealized summary of hepatic *O*-demethylase activity as related to ascorbic acid deficiency and dietary dieldrin. Average values for all experiments in each clinical period of the development of scurvy are plotted at the midpoints of these periods. Each point in the period of normal weight gains is the mean of 20–24 animals and represents the data presented in Fig. 1 and Table 3. Values for the period of depressed gains were derived from the data presented in Table 2 and values for the period of frank scurvy are from the data in Table 1.

In order to appraise the adequacy of recommended dietary levels of ascorbic acid in maintenance of inductive capacity, guinea pigs were fed various dietary levels of the vitamin. All animals were placed on ascorbic acid-deficient diet for 8 days. During the following 4 days the magnitude of induction by 25 ppm dieldrin was compared in guinea pigs fed 50, 200, or 2000 ppm ascorbic acid. The results are summarized in Table 5. There was little induction of either enzyme in animals fed 50 ppm ascorbic acid and even the induction of these enzymes in animals fed 200 ppm ascorbic acid was less than in those fed 2000 ppm of the vitamin. Similar trends had also been seen in an earlier pilot experiment of the same design. In both of these experiments liver concentrations of ascorbic acid were nearly equal in animals fed 50 and 200 ppm ascorbic acid.

TABLE 6
AMINO ACID COMPOSITION OF SKIN COLLAGEN RESIDUES/1,000 TOTAL RESIDUES

Amino Acid	Guinea Pig Skin Collagens			Calf Skin Tropocollagen[46]
	Neutral Salt Soluble	Acid Soluble	Total Collagen	
Hydroxylysine	5.0	5.6	4.9	5.6
Ornithine	...	...	2.5	2.5
Lysine	30	28	31	26.9
Histidine	6.0	5.9	4.8	4.7
Arginine	50	49	47	48.7
Cysteic acid	0.9	0.5	0.7	trace
Hydroxyproline	91	91	94	93.7
Aspartic acid	59	53	47	50.2
Threonine	18	19	19	19.0
Serine	35	36	40	39.4
Glutamic acid	70	71	74	79.5
Proline	119	128	119	120.8
Glycine	332	330	323	337.9
Alanine	108	109	113	108.9
Valine	24	22	17	14.6
Methionine SO_2	7	7.6	10	...
Isoleucine	11	11	10	9.1
Leucine	24	23	24	27.1
Tyrosine	2.4	1.8	3.5	4.5
Phenylalanine	12	12	14.6	12.8
Proline/ Hydroxyproline	1.3	1.4	1.3	1.3

Discussion

The collagen extracted with neutral salt is considered to be an early form of collagen fibrils[11–13,27,28] but lacks the necessary intermolecular cross-linking required for insolubility. The highest specific activity was found in this neutral salt-soluble collagen (Table 1) for both proline and hydroxyproline regardless of treatment corroborating the earlier data. Recently, Tsurufuji and Nakagawa[29] presented data on the incorporation of ^{3}H-proline into rat-skin salt, acid, and insoluble collagen, which they interpret to mean that the majority of neutral salt-soluble collagen matures into acid-soluble and insoluble collagen. Nimni[26] reported the accumulation of radioactive proline in the salt-soluble fractions of rat skin in the first 24 hours to almost the exclusion of the acid-soluble and the insoluble collagen, again indicating that the salt soluble collagen is the first synthesized.

The neutral salt extractable collagen is clearly the first synthesized of the collagen fractions which were extracted, but the next progression of the label into the acid-soluble or insoluble collagen could not be determined. Piez,[28] following the chromatographic characteristics and amino acid composition of α_1, α_2 and α_3 collagen, has diagrammed a possible representation of the cross-linking process of collagen.

The extent of hydroxylation may be determined by the ratio of the specific activity of hydroxyproline to proline (Table 3). According to the manufacturer, the proline used in these experiments was labeled uniformly with tritium for all positions of hydrogen, with an average of nine tritium atoms/molecule prior to incorporation into peptide linkage and an average of seven tritium atoms after peptide bond formation. If one of the seven tritium atoms was exchanged during hydroxylation and not reincorporated, the recovered radioactivity would be six atoms of tritium or a theoretical average of 86%. The conversions found were 78% to 80%, which would give values of 5.5 and 5.6 tritium atoms recovered in the hydroxyproline. Since recoveries tend to be low and since the uniformity of the label distribution is not known, this in vivo incorporation would corroborate previous in vitro data[30,31] for the exchange of one tritium from 3,4 ^{3}H-proline and the loss of one tritium from the four position in the conversion to hydroxyproline.[32]

Since a value of 86% is approached only in the total collagen that consists predominantly of insoluble collagen for all animals

be that the inducing agent can reverse to a limited extent cellular priorities for the deficient nutrient. An alternative speculation about the diminution of the ascorbic acid-deficiency-dieldrin interaction during the 12- to 14-day period is that different mechanisms produce the interaction during the early feeding period and later in frank scurvy. During the intermediate period the two processes may tend to produce an apparent cancellation of effects.

Maintenance of inductive capacity appears to require greater quantities of ascorbic acid than prevention of scurvy. Ginter *et al.* (1968) maintained guinea pigs in a scurvy-free state for 1 year on a level of the vitamin equivalent to a diet containing 25 ppm ascorbic acid. But, as reported here, a diet with double this amount of ascorbic acid did not support induction. Even animals fed the minimum dietary level of 200 ppm ascorbic acid recommended by the National Research Council (1964) were not able to support maximum induction.

Maintenance of induction appears to be more directly dependent on dietary levels of ascorbic acid than on concentration of the vitamin in the liver. Animals fed 50 ppm ascorbic acid maintained about the same amount of the vitamin in the liver as those fed 200 ppm, yet had much lower enzyme activity. This would indicate that a constant dietary supply of the vitamin would be more effective than one which fluctuated.

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Trial of Ascorbic Acid in Prevention of Colds

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Ascorbic acid is widely used as a prophylactic and therapeutic agent against the common cold, and is in fact often recommended for this purpose by medical practitioners. It may be administered as a large dose as soon as possible after the first symptoms are felt, and this dosage is then continued for a few days in order to "abort" the cold. Though many people use this treatment on themselves and believe it is effective, it is impossible to assess its efficacy. Untreated subjects may experience symptoms such as sneezing or a mild sore throat, which then disappear without going on to a full cold, and patients' assessments of their colds are much influenced by any treatment owing to the placebo effect (Diehl *et al.*, 1938).

In these studies we attempted to show by in-vitro experiments that exposure of cells to ascorbic acid increased their resistance to infection with viruses. Though there was no activity in vitro, we also attempted to demonstrate a protective effect in animals or man, since it was thought that host resistance might be enhanced indirectly.

Materials and Methods

Virus Strains.—Strains of viruses selected from the biological groups known to cause common colds and related diseases were used (see Table I).

* From the Common Cold Research Unit, Salisbury, Wilts.

Tissue Cultures.—Roller-tube cultures were used, as follows: (1) "Diploid" human embryo lung fibroblast cells (HEL). A semicontinuous line isolated in this laboratory was used and maintained in Eagle's medium with 2% ox serum and antibiotics. (2) Monkey kidney cells (MK); cultures of secondary trypsinized cells were washed three times and maintained in Eagle's medium. (3) Hela cells of the Bristol line were maintained in 2% rabbit serum in Eagle's medium.

Ascorbic Acid.—Ascorbic acid for injection was added to tissue culture medium immediately before use. Preliminary experiments showed that 10 mg. of ascorbic acid/100 ml. of medium was not toxic in HEL, HeLa, or MK cells, and there was no crystal deposition. This concentration was therefore

TABLE I.—*Viruses Used in Tissue-culture Experiments with Ascorbic Acid*

Family	Group	Strain	Tissue Used for Propagation
Picornavirus ..	Rhinovirus H	16/60	Human embryo lung
" ..	" H	EB	" " "
" ..	" M	HGP	" " "
Myxovirus ..	Influenza B	B Eng/101/62	Monkey kidney
" ..	Paraintluenza 3	Prototype	" " "
" ..	Respiratory syncytial	Randall	HeLa
Adenovirus ..	Adenovirus 5	Prototype	"
Enterovirus ..	Poliovirus 1	LSc 2ab	Human embryo lung
" ..	E.C.H.O. 11	U virus	" " "
" ..	Coxsackie A21	Local strain	" " "
Herpesvirus ..	Herpes simplex	Petrett*	" " "

* Albanese *et al.* (1966).

used in all the tissue-culture work. The volunteers took 1 g. of ascorbic acid three times a day as effervescent tablets supplied by Roche Laboratories. These were given for three days before virus inoculation, and for six days after. No side-effects of the treatment were observed. Control subjects were given placebo tablets which were indistinguishable from the ascorbic acid tablets except by chemical analysis.

Volunteers.—These were housed and observed as described elsewhere (Tyrrell, 1965). They ranged in age from 18 to 50 (mean 30.2 years). They received a generous mixed diet which included fresh fruit and cooked vegetables. The dietary ascorbic acid was not measured, but the trials were conducted when the general dietary intake both before and during the trials was expected to be low, so that the effects of supplements would be expected to be maximal. Two serum specimens were taken from most volunteers; the first before virus inoculation, and the second about a fortnight after inoculation. They were tested for antibodies against the virus with which the volunteer had been inoculated by neutralization in tissue culture for rhinoviruses and by haemagglutination inhibition for influenza. Nasal washings were collected daily from the volunteers for five days, from the first day after virus inoculation. Phosphate buffered saline was used to take the washings, and the specimens were either inoculated immediately into tissue-culture tubes or mixed with an equal volume of nutrient broth, stored at −70° C. and tested later. Representative viruses isolated from the washings were typed by neutralization tests with specific antiserum. Volunteers were inoculated with a virus which had been propagated by serial passage in volunteers. The virus was stored as nasal washing at −70° C. and thawed and diluted 1/10 in Hanks's saline immediately before use.

Mice.—Porton strain white mice were used. They were given 4 mg. daily of a freshly prepared solution of ascorbic acid in saline or of plain saline by intraperitoneal injection for six days before and nine days after virus inoculation. Influenza A (Mel) virus was administered as intranasal drops under light ether anaesthesia.

Results

Experiments in Tissue Cultures

Table II shows the results of experiments in which cells in tissue-culture tubes were exposed to medium containing ascorbic acid for one to one and a half hours before being inoculated with viruses. After inoculation the culture tubes were rolled at 33° C. and observed daily for a cytopathic effect or, in the case of tissue infected with a myxovirus, tested several days later by haemadsorption. The minimum infectious dose for each virus was the same in treated and in untreated cultures. However, it was shown that there was a marked decrease in the ascorbic acid content of the medium within four hours of its addition to medium whether in contact with cultures or not. In one experiment with adenovirus type 5 the medium was therefore changed every 12 hours for four days, but there was still no apparent antiviral effect.

TABLE II.—*Minimal Infectious Dose of Virus for Tissue Cultures Maintained in Medium Containing Ascorbic Acid*

Virus	Infectious Dilution (TCD_{50}) in Titrations Performed in Cultures Treated with	
	Ascorbic Acid	No Ascorbic Acid
Rhino M	10^{-4}	10^{-3}
" H (16/60)	10^{-3}	10^{-3}
" H (FEB)	10^{-4}	10^{-4}
Polio 1	$10^{-3.5}$	10^{-5}
E.C.H.O. 11	$10^{-3.5}$	10^{-6}
Coxsackie A21	10^{-4}	10^{-4}
Influenza B	10^{-3}	10^{-3}
Parainfluenza 3	10^{-4}	$10^{-4.5}$
Respiratory syncytial	10^{-2}	$10^{-1.5}$
Adeno 5	10^{-2}	$10^{-1.5}$
Herpes	10^{-5}	10^{-5}

Experiments on Mice

Mice were treated with intraperitoneal ascorbic acid at a dose of about 300 mg./kg. As shown in Table III, neither the mortality rate nor the extent of the lung lesions in the survivors was reduced.

TABLE III.—*Results in Mice Treated with Ascorbic Acid and Challenged with Influenza A Virus*

Treatment		Dilution of Virus	
		10^{-3}	10^{-4}
Ascorbic acid	Lung lesion score*	12/16	3/16
	Deaths	2/4	0/4
Saline	Lung lesion score	6/16	3/16
	Deaths	1/4	0/4

* Slightly modified from the method of Horsfall (1939).

Experiments on Volunteers

The experiments took place in the first five months of 1966. Volunteers received 3 g. daily—that is, about 75 mg./kg./day—of ascorbic acid by mouth for three days before being challenged with a small dose of virus which had been passed from man to man. Table IV shows the number of volunteers who developed colds, and Table V the results of virus isolation studies and of antibody titrations on acute and convalescent sera.

The viruses used were drawn from all those known to be frequent causes of colds. The rhinoviruses were represented by three strains, two of which belonged to the M and H types, the third, H.S., being a recently described type which can be cultivated only in organ cultures. The B814 virus is a recently cultivated virus which is now known to be morphologically similar to avian bronchitis and to at least one other virus causing human colds (Almeida and Tyrrell, 1967); the influenza B virus is a typical myxovirus, and influenza and parainfluenza viruses cause an appreciable proportion of colds, particularly in

TABLE IV.—*Clinical Response of Volunteers who received Ascorbic Acid Tablets and were then Inoculated with Viruses Capable of Causing Colds*

	Results in Volunteers Receiving							
	Rhinoviruses		Influenza B Virus		B814 Virus		All Viruses	
	Ascorbic Acid	Placebo	Ascorbic Acid	Placebo	Ascorbic Acid	Placebo	Ascorbic Acid	Placebo
Number inoculated	29	26	8	8	10	10	47	44
Number of colds	9	9	4	4	5	5	18	18
Mean incubation period (days)	3·1	2·4	3	2·8	3	3·8	3	2·9
Clinical severity of colds: Mild	7	7	1	1	3	3	11	11
Clinical severity of colds: Moderate	—	1	—	1	2	—	2	2
Clinical severity of colds: Severe	2	1	3	2	—	2	5	5
Mean duration of colds (days)	7·4	9·2	10·7	8·5	6·6	4·6	8	8
Mean score*	6·5	5·9	13·7	9·2	6·2	7·0	8	7
Paper handkerchiefs used daily at peak of cold: Range	4–32	3–56	6–82	10–23	8–25	7–38	4–82	3–56
Paper handkerchiefs used daily at peak of cold: Mean	10	14	35	17·5	14	10·8	16·5	16·5

* The score is calculated by allotting points for both the number of days on which individual symptoms and signs were recorded and the severity of each.

young children. If ascorbic acid had a general prophylactic effect on colds it should have had an effect on colds produced by rhinoviruses and probably also by the other viruses used.

Table IV shows the results obtained. It indicates that the number of colds and their severity were not affected by ascorbic acid. The length of the incubation period and the duration of symptoms were variable, but the mean values of both these periods were the same in treated and untreated volunteers. In parallel with these experiments 18 volunteers received non-infectious intranasal drops and two developed a cold. In the case of two rhinoviruses and the influenza B virus it was possible to test for virus infection, and Table V shows that virus infection was not reduced in frequency by the treatment. Naturally, the volunteers who were infected were drawn from the group who had low antibody titres and the number of these in each trial varied unpredictably, but there was no evidence that subjects with little or no antibody were protected from infection by ascorbic acid. We considered the possibility that the results described in the literature might be due to a rather specific effect of ascorbic acid on either the symptoms of sore throat or the occurrence of mucopurulent nasal discharge. Thirteen volunteers who developed colds while taking ascorbic acid complained of sore throat, while 15 who were on placebo tablets did so. The comparable figures for mucopurulent nasal discharge were 10 and 8. It was concluded that there was no evidence for a general, antiviral, or symptomatic prophylactic effect of ascorbic acid in these experiments.

TABLE V.—*Results of Laboratory Tests on Volunteers Inoculated with Three Different Viruses*

	Results in Volunteers Given									
	Ascorbic Acid					Placebo				
	Total Volunteers	Colds	Virus Isolated	Antibody Rise	Laboratory Evidence of Infection	Total Volunteers	Colds	Virus Isolated	Antibody Rise	Laboratory Evidence of Infection
M Rhinovirus PK	15	5	5	6	9	16	3	5	2/14	5
H „ DC	6	2	4	5	6	3	2	2	3	3
Total rhinoviruses	21	7	9	11	15	19	5	7	5/17	8
Influenza B ..	8	4	5	2	5	8	4	6	1	6

In two trials the volunteers were told whether or not they had been given ascorbic acid before their colds had finished, and the colds in the treated groups were appreciably shorter than in the controls; in the third and later trials they were not told which they had received, and the duration of the colds was similar in both groups. This could have been due to the effect of ascorbic acid or to coincidence, but may have been a result of therapeutic suggestion.

We wished to confirm that the vitamin was being taken and absorbed properly. Therefore, in the trial in which influenza B was used a urine sample was collected from each volunteer during the incubation period. This was titrated for its ascorbic acid content by the dichlorophenol-indophenol method. No ascorbic acid was detected in the urine of nine volunteers receiving placebo, and from 28 to 126 mg./100 ml. (mean 73 mg./100 ml.) was found in eight receiving the vitamin.

Discussion

In reading the literature it has been noted that conflicting claims have been made regarding the value of vitamin C either alone or in combination with bioflavonoids in the prevention and treatment of acute infections of the upper respiratory tract.

Uncontrolled observations are of course impossible to evaluate, but even where placebos are used as a basis for comparison there have been many important differences between the studies reported; they have differed in the type of clinical illness occurring in the population, the day and method of clinical assessment, the age and nutritional state of the subjects, and the amount of vitamin given and whether it was administered prophylactically or therapeutically. One trial, for instance, suggested that there was a marked reduction in the duration of colds in baseball players given prophylactic vitamin C (Barnes, 1961), but the control group observed were not players.

In another controlled trial there was an apparent improvement two days after treatment in subjects given a mixture of bioflavonoids, ascorbic acid, and aspirin rather than another analgesic capsule (Macon, 1956); it is impossible, however, to decide which of the differences in medication accounted for the results. In another large trial (Tebrock *et al.*, 1956) a mixture of bioflavonoids and of 200 mg. of ascorbic acid daily was given after the onset of the cold, but the clinical observations, which were made only one and three days after treatment began, showed no evidence of improvement. Other smaller trials also showed no effect. Banks (1964, 1965) has drawn attention to the possibility that treatment with ascorbic acid may reduce the number of colds which are followed by long-continued purulent discharge. Table VI summarizes some facts from what seem to us to be among the most critically evaluated of the earlier trials; this supports the view that small doses of ascorbic acid administered to those on a low intake or large doses administered to those on a normal intake may reduce the duration of pharyngitis or colds.

TABLE VI.—*Some Previous Controlled Studies on the Effect of Ascorbic Acid on Length of Respiratory Infections*

Population Studied	Type of Illness	Dosage of Ascorbic Acid	Effect Observed
20 volunteers on scorbutogenic diet*	Colds	Continuous 10 or 70 mg. daily	Colds in deprived group lasted 6·4 days (G.M.), in non-deprived 3·3 days. Not statistically significant
Adolescents in institution with low vitamin C intake†	Tonsillitis (probably streptococcal) and colds	Continuous 200->50 mg. daily	No effect on incidence. Tonsillitis shorter in treated groups. Colds unaffected
Members of ski camp‡	Pharyngitis (colds apparently uncommon)	Continuous 1,000 mg. daily	Incidence reduced

* Bartley *et al.* (1953). † Glazebrook and Thomson (1942). ‡ Ritzel (1961).

We thought it to be intrinsically unlikely that ascorbic acid could influence the uptake or replication of viruses. Nevertheless, the "uncoating" of viruses and the damage they produce may be mediated by the release of enzymes from lysosomes (Mallucci and Allison, 1965), and these organelles may be stabilized by ascorbic acid—at least in photosensitized epithelial cells (T. F. Slater, personal communication, 1966). It was therefore possible that the vitamin might influence the multiplication and cytopathic effect of viruses. The results of the in-vitro experiments were negative, but were not entirely convincing because of the rapid rate of inactivation of the vitamin in the cultures used. Since ascorbic acid can apparently improve the response to stress of the rat and influence the repair of skin, it might assist repair of the mucosa from the damage produced by viruses. It was therefore thought justifiable to undertake volunteer experiments with large doses of ascorbic acid administered before a known virus and a strict double-blind procedure to evaluate the clinical effects; laboratory techniques were used to determine, when possible, which volunteers had antibodies and which were infected.

We conclude that there is no evidence that the administration of ascorbic acid has any value in the prevention or treatment of colds produced by five known viruses. The literature indicates that the administration of the vitamin to deficient subjects may reduce the incidence or severity of colds and possibly of pharyngitis due to streptococci, as well as the incidence of rheumatic fever and pneumonia (Glazebrook and Thomson, 1942). However, it is quite reasonable to conclude

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that administering very large doses of vitamin to well-nourished people does not have the same effect.

It is sometimes said that the colds produced at this unit are not comparable to those which occur in people who are exposed to natural infection and who may develop hypothetical secondary bacterial infections. We, on the other hand, are impressed with the fact that the colds observed here experimentally are very similar clinically to those, caused by the same viruses, which are observed in clinical practice (Tyrrell, 1965), and we should be surprised if ascorbic acid had any prophylactic or therapeutic value for well-nourished subjects who caught colds at home or at work.

Summary

Preliminary experiments showed no evidence that ascorbic acid protected cultures against several respiratory viruses or white mice against influenza. In further experiments 91 volunteers were given either 3 g. of ascorbic acid daily or placebo tablets. Three days after the treatment began they received intranasal drops containing an M rhinovirus, an H rhinovirus, a "new" rhinovirus which grows only in organ cultures, influenza virus type B, or a "new" virus related to avian bronchitis. The number, duration, and severity of the resulting colds were the same in the treated and control groups. There was no evidence that colds produced by any single virus were favourably affected.

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Inhibition of Hexosediphosphatase by Sulphhydryl Reagents and by Ascorbic Acid

DURING the course of an investigation of the action of vitamin K and its biological antagonists (dicoumarol, salicylate) on various phosphatases, details of which will be published elsewhere, it was observed that liver hexosediphosphatase[1] exhibited a much greater sensitivity towards quinones than did the other phosphatases. Purified bone phosphomonoesterase[2], for example, required a concentration of 10^{-3} M benzoquinone for complete inactivation, whereas 10^{-5} M benzoquinone was sufficient completely to inhibit the hexosediphosphatase.

It is well known that enzymes the activity of which is dependent on free —SH groups are inhibited by quinones in very low concentrations, and it has been suggested that the antibiotic activity of quinones is a result of this effect[3]. With the exception of some pyrophosphatases and transphosphorylases, the phosphatases, the activity of which is probably dependent on free amino-groups[4], are, on the other hand, not affected by —SH reagents[5]. The comparatively high quinone sensitivity of hexosediphosphatase, however, led us to examine the behaviour of this enzyme towards other substances which are known to react with thiol groups.

Our experiments show that hexosediphosphatase is inhibited by iodoacetate, by phenyl mercuric nitrate and by alloxan. Cysteine, which itself inhibits in sufficiently high concentration but strongly activates at lower concentrations, completely reverses the iodoacetate and phenyl mercuric nitrate inhibitions and partly reverses that by alloxan. Thus inhibition by iodoacetate (10^{-2} M) or by alloxan ($2 \cdot 5 \times 10^{-3}$ M) is 40 per cent. Cysteine (10^{-2} M) reactivates the iodoacetate-inhibited enzyme 100 per cent and the alloxan-inhibited enzyme 37 per cent (that is, inhibition is reduced from 40 to 25 per cent). Inhibition by alloxan (5×10^{-3} M) is complete and there is no reactivation by cysteine. Phenyl mercuric nitrate (10^{-4} M) inhibits 100 per cent, with complete reactivation by cysteine ($2 \cdot 5 \times 10^{-4}$ M). Cysteine (5×10^{-4} M and 1×10^{-3} M) alone activates 98 and 96 per cent respectively, while an increase in concentration to 5×10^{-3} M results in complete inactivation.

The hexosediphosphatase used was prepared as described by Gomori[1] and was free from non-specific phosphatase activity. Mixtures (10·0 ml.) of the enzyme solution (0·1 ml.), borate buffer (2·0 ml.), 0·05 M magnesium sulphate (2·0 ml.), sodium hexosediphosphate solution (2·0 ml. ≡ 0·2 mgm. phosphorus) and inhibitor were incubated for 30 min. at 37° and pH 9·1 in separate tubes, and the inorganic phosphate in each was determined colorimetrically. The quantity of enzyme used was such that half the substrate was hydrolysed in the controls (that is, no inhibitor) during the period of incubation. No phosphate was liberated in the absence of enzyme. When iodoacetate was used, it was incubated with the diluted enzyme-magnesium-buffer mixture for 15 min. before addition of the substrate and of cysteine.

Experiments with ascorbic acid, which was expected to activate the enzyme by virtue of its reducing properties, showed it to be a very powerful inhibitor. Concentrations below 5×10^{-7} M resulted in slight activation (up to 5 per cent), but concentrations greater than 10^{-6} M inhibited, with complete inactivation in the presence of 2×10^{-3} M ascorbic acid. This inactivation by ascorbic acid is undoubtedly due to competition with the hexosediphosphate, as indicated by the results of experiments with varied substrate concentrations. Results of a typical experiment are given in the accompanying table.

Ascorbic acid, molar concentration × 10^4	Substrate solution (ml.)	Inorganic phosphorus (μgm.)	Inhibition (per cent)
0	1·5	573	0
2·5	1·5	371	35
5	1·5	317	45
5	3·0	245	32
10	3·0	200	45
0	3·0	360	0

These results also confirm Gomori's observation that the substrate itself inhibits the enzyme as the concentration is raised and, for this reason, the amount of enzyme solution used was increased to 0·15 ml. in the above experiment.

Inhibition of β-malt amylase by ascorbic acid and related compounds was attributed by Hanes[6] to the presence in the molecule of the di-enol grouping. Giri[7] has shown that several plant and animal phosphatases, which were not affected by ascorbic acid, were inhibited by an ascorbic acid copper complex. Our experiments demonstrate that ascorbic acid may

compete for an enzyme with a substrate which it resembles in ring structure. The possibility that ascorbic acid may compete with hexose diphosphate for other enzymes such as aldolase or with other furanose-sugar phosphates, and thereby influence the course of carbohydrate or nucleotide metabolism, seems to us to be worthy of further investigation.

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