SYNTHESIS AND SOME MAJOR FUNCTIONS OF VITAMIN C IN ANIMALS *

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The requirement of ascorbic acid (vitamin C) is a common property of living organisms, and it has long been considered that all animals except the guinea pig, monkey, and man can synthesize this vitamin. The classic method for determining the ability of an animal to synthesize ascorbic acid is to feed it a scorbutogenic diet for a prolonged period and to observe the appearance of the scurvy syndrome. Obviously, the method is laborious and time-consuming. Also, the onset of the scorbutic syndrome depends on the ascorbic-acid-retention capacity of the animal. For example, whereas the guinea pigs can be made scorbutic in about 3 weeks, it takes 3 to 4 months to produce scurvy in man. Since the discovery of the technique for studying ascorbic acid synthesis *in vitro*,[1-8] the task has become much simpler. In this technique, the tissue homogenates or the subcellular fractions are incubated with precursors of ascorbic acid and the amount of the vitamin formed is estimated. Using the *in vitro* method, we have examined the ascorbic acid synthesizing abilities of different species of animals in the phylogenetic tree, and the results are given below.

Evolution and Biosynthesis of Ascorbic Acid

The ability to synthesize ascorbic acid is absent in the insects, invertebrates, and fishes. The biosynthetic capacity started in the kidney of amphibians, remained in that of reptiles, became transferred to the liver of mammals, and finally disappeared from the guinea pig, the flying mammals, the monkey, and man. A similar transition in the biosynthetic ability was observed in the branched evolution of birds.[9-13] Table 1 shows ascorbic acid synthesis from L-gulono-1,4-lactone in microsomal fraction from tissues of different species of animals. Figure 1 shows how the overall pattern of ascorbic acid synthesis by different species of animals is correlated to their phylogeny.

Evolution of the Biosynthetic Capacity

The incapability of insects, invertebrates, and fishes to synthesize ascorbic acid apparently raises the question whether ascorbic acid is an essential requirement for these species. It has been reported that salmon, trout,[14, 15] and the desert locusts [16] are dependent on dietary ascorbic acid. However, the need of

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TABLE 1

ASCORBIC ACID SYNTHESIS FROM L-GULONO-1,4-LACTONE IN MICROSOMAL FRACTIONS FROM TISSUES OF DIFFERENT SPECIES OF ANIMALS *

Animals	Ascorbic Acid Synthesized (μg/mg Protein/Hr) Kidney	Liver
Insects † ‡	—	—
Invertebrates † ‡	—	—
Fishes †	—	—
Amphibians		
Toad (*Bufo melanostictus*)	144±10	—
Frog (*Rana tigrina*)	115±10	—
Reptiles		
Turtle (*Lissemys punctata*)	98±8	—
Bloodsucker (*Caloter versicolor*)	50±5	—
House lizard (*Hemidactylus flaviviridis*)	46±6	—
Common Indian Monitor (*Varanus monitor*)	32±4	—
Angani (*Mabuya carinata*)	25±4	—
Snake (*Natrix piscator*)	18±2	—
Tortoise (*Testudo elegans*)	14±2	—
Mammals		
Goat	—	68±6
Cow	—	50±6
Sheep	—	43±4
Rat	—	39±4
Mouse	—	35±4
Squirrel	—	30±4
Gerbil	—	26±4
Rabbit	—	23±2
Cat	—	5±1
Dog	—	5±1
Guinea pig	—	—
Flying mammals		
Indian fruit bat (*Pteropus medius*)	—	—
Indian pipistrel (*Vesperugo abramus*)	—	—
Primates		
Monkey (*Macaca mulatta*)	—	—
Man	—	—

* For incubation and other detail conditions see Reference 13. Each datum represents an average from a minimum of 8 animals ± S.D. In case of house lizards, kidneys from 12 lizards were pooled for 1 determination, and 4 such determinations were made.

† Accounts of insects, invertebrates and fishes have been given elsewhere.[11, 12]

‡ In cases of insects and invertebrates, homogenates of the fat body or the hepatopancreas and the malpighian tubules were used in place of liver and kidney microsomes.

insects, invertebrates, and fishes for ascorbic acid may be very small, and they may obtain enough vitamin through food. Naturally, there was no necessity to synthesize the vitamin, and the biosynthetic mechanism did not evolve in these earlier species of the phylogenetic tree.

The emergence of the biosynthetic ability in the amphibians suggests that a

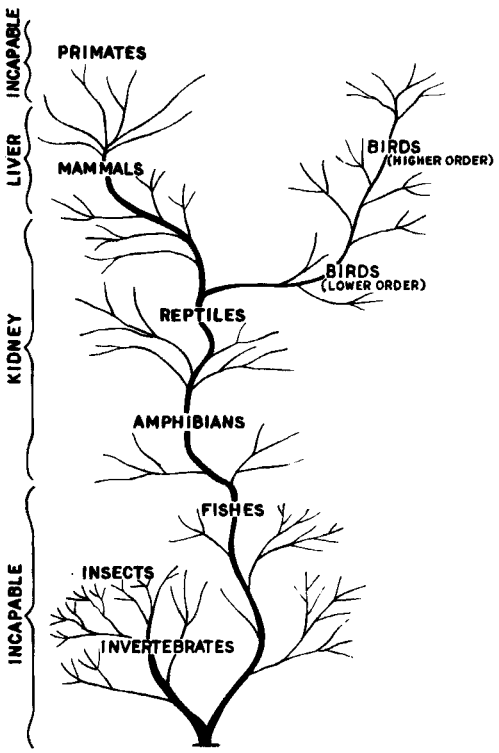


FIGURE 1. Schematic representation of ascorbic-acid-synthesizing abilities of various species of animals in relation to their phylogeny. (From Chatterjee.[13] By permission of *Science*.)

greater need of the vitamin was somehow linked with the evolution of vertebrates from the aquatic to the terrestrial environment. The evolution of all lines of vertebrates generally required loss of specialization as new specialization came up. The evolution of ammonotelic fishes to the ureotelic amphibians is associated with the newly specialized capacity to synthesize ascorbic acid. Whereas the fishes are unable to synthesize the vitamin, kidney homogenate from the water-living tadpole synthesized a significant amount of ascorbic acid *in vitro* (200–250 μg/g kidney/hour).

When the descendants of certain crossopterygian fishes ventured out of the water and crawled upon the mud banks of streams and lakes at the end of Devonian period to become the first amphibians,[17] the vertebrates entered a completely new course of evolutionary development. The step from the aquatic to the terrestrial mode of life was a profound change involving a tremendous range of adaptations under strong selection pressure. The primitive quadrupedal vertebrates had to face a variety of extreme stressful conditions such as support of body weight, locomotion on land against gravity, high oxygen tension, and desiccation by dry air and hot sun. Perhaps the requirement of ascorbic acid was a must for overcoming the stressful conditions and the species with the biosynthetic ability survived competition. The beneficial effect of ascorbic acid in stress is now a well-established fact.[18, 19]

It is difficult to determine why ascorbic acid is synthesized in the kidney and not the liver of the amphibians. It may be considered that the early amphibians started synthesis in an organ where the vitamin could be produced at a high rate. In fact, the *in vitro* results (TABLE 1) show that in frogs and toads, the activity of the kidney enzyme is much higher than that of the mammalian liver enzymes. The K_M for the frog and toad kidney microsomes was approximately 1 mM, whereas that for the liver microsomes from mammals was in the range of 4–5 mM.

Transition of the Biosynthetic Capacity from Kidney to Liver

The amphibians' capacity to synthesize ascorbic acid in the kidney continued in that of reptiles but was transferred to the liver of mammals. The change of the site of synthesis from the kidney of reptiles took place when the vertebrates were evolving temperature regulatory mechanism and changed from cold-blooded forms to the warm-blooded species. The activity of the kidney tissues had been altered to accommodate the necessities of life on dry land with increased physiological demands such as regulation of urea, calcium, phosphate, and other ions. It is possible that the relatively small kidneys became too crowded with these demands.[20]

Though the activity of L-gulono oxidase (EC No. 1.1.3.8.) of kidney microsomal fractions from reptiles is comparable to that of the liver microsomal fractions from mammals (TABLE 1), yet the net synthesis of ascorbic acid per kg body weight per day is comparatively very much lower in reptiles.[13] This is because the weight of the kidney of reptiles is relatively small, only about 0.13–0.4% of body weight, whereas the liver of mammals comprises about 4–5% of body weight. One explanation that may be given for the transition of the site of synthesis from the kidney of reptiles to the liver of mammals is that the mammals need more ascorbic acid than the reptiles for detoxification of histamine.[13] And to get more ascorbic acid, probably the site of synthesis was transferred to the much larger liver in the course of evolution.

Synthesis in Birds

In contrast to the evolution of mammals from the synapsids, the birds are believed to have evolved from a quite different line of reptiles, the archosaurian-stem reptiles.[21] The birds retained outstanding reptilian features in the jaws, the cranium, and even the brain.[22] This similarity is further evident in the fact that the primitive birds retained the biosynthetic capacity in the kidney. With the progress of evolution, the biosynthetic capacity of the kidney is shared by the liver of 2 passeriform birds, the house crow and the common myna.[5, 10] These birds may be considered transitional, on the borderline in transferring the biosynthetic capacity from kidney to liver. Thereafter, the ascorbic-acid-synthesizing capacity was taken over by the liver of more evolved passeriform birds, while a number of other highly evolved Passeres are incapable of producing the vitamin.[10] The pattern of ascorbic acid synthesis in birds is thus similar to that in the mammalian line of evolution (FIGURE 1).

Loss of the Biosynthetic Ability

The biosynthetic capacity is lost in the guinea pig, the flying mammals, the monkey, and man. The flying mammals are considered to be more near to the primates. The absence of biosynthetic capacity in this species supports the contention. The inability of the guinea pig may be explained by the consideration that the mutation in the guinea pig occurred independently.

The results obtained with birds as reported before[10] indicated that the activity of the microsomal enzyme L-gulono oxidase was comparatively higher in the primitive birds. In the more evolved passeriform birds, the enzymic activity decreased until it became minimum in the jungle babbler (*Turdoides somervillei*[10]), after which the other Passeres were shown to be incapable of synthesizing the vitamin. Similarly, in the mammals the activity of L-gulono oxidase became minimum in more evolved cats and dogs (TABLE 1) and then disappeared from the guinea pig, the flying mammals, and other primates.

The failure of the guinea pig, the flying mammals, monkeys, and man to synthesize ascorbic acid is due to a common defect, namely, the absence of the terminal enzyme L-gulono oxidase.[4, 9, 23] This in turn may be attributed to the loss of the gene or the capacity of the gene responsible for synthesizing the enzyme. While the biosynthetic capacity started in the amphibians, which evolved roughly about 350 million years ago, the gene mutation leading to loss of the capacity probably took place in the common ancestor of man and other primates roughly about 25 million years ago.[24] The mutation leading to the loss of such an essential gene was however, neutral[25] and not lethal. The mutants did not become extinct because the environment furnished the vitamin and the species continued to survive.

Apparently, the loss of ascorbic-acid-synthesizing ability in the progress of evolution is a disadvantageous mutation. However, in the consideration of Pauling,[26] man and other primates have an advantage over the ascorbic-acid-producing animals in that they have been relieved of the burden of constructing and operating the machinery for production of the vitamin. From the neuroanatomical and behavioral point of view, there is no doubt that the higher primates surpass all other animals in intelligence, and man is at the head of all. Is there any correlation between the loss of ascorbic-acid-synthesizing ability and the gain of superior intelligence?

INDUCED HISTAMINE FORMATION AND BIOSYNTHESIS OF ASCORBIC ACID IN THE RAT

It has been shown that various drugs and carcinogens markedly induced the synthesis of ascorbic acid in the rat, as revealed by its enhanced urinary excretion.[27, 28] However, the mechanism of this induction was not clear. It is known that the drugs concerned also increase the activities of the microsomal drug-metabolizing enzymes.[29] Therefore, the possibility may exist that the enhanced excretion of ascorbic acid under the influence of drugs and carcinogens is due to an increase in the activities of the various enzymes operative in ascorbic acid synthesis. Conney et al.[28] reported that pretreatment of rats with chlorobutanol did not stimulate any of the enzyme systems involved in the biosynthetic pathway except that the UDPG dehydrogenase activity was increased about twofold. However, Touster and Hollman[30] showed that while 3,4-benzpyrene

and 3-methyl cholanthrene stimulated ascorbic acid synthesis significantly, the carcinogens did not enhance the activity of UDPG dehydrogenase in the rat liver. Moreover, Aarts [31] had shown that a single administration of chlorobutanol to rats enhanced the synthesis of ascorbic acid markedly but UDPG dehydrogenase activity was not increased. Salomon and Stubbs [32] also reported that ascorbic acid synthesis by chlorotone could not be accounted for only by an increased UDPG dehydrogenase activity and that possibly a different mechanism existed.

In view of the wide variations in structure and pharmacological properties of the various drugs and carcinogens that stimulated ascorbic acid formation in the rat, we considered that the different compounds probably produce *in vivo* a common response to the system that in turn would lead to an induced formation of ascorbic acid. Recently we observed an incidental relationship between stimulation of ascorbic acid synthesis and enhancement of histamine formation.

The results presented in TABLE 2 indicate that, irrespective of the structure and pharmacological properties of the various drugs and chemical compounds administered, whenever there was an induction of histamine-forming capacity (HFC) in the rat liver, there was an induction in ascorbic acid biosynthesis as revealed by the elevated urinary level and increased liver content of the vitamin. Compounds that did not induce HFC did not stimulate ascorbic acid production (footnotes to TABLE 2).

TABLE 2

BIOSYNTHESIS OF ASCORBIC ACID AND INDUCED FORMATION OF HISTAMINE IN RAT LIVER

Treatment	Dosage/100g Body Wt./Day	HFC in ng/ g Liver/90 Minutes *	Mg Urinary Ascorbic Acid/ Rat/Day *
None	—	62±3.5	0.65±0.02
Phenobarbital	2.5 mg	202±6.4	7.40±0.02
Chlorobutanol	20 mg	196±5.7	6.1 ±0.02
Chlorpromazine	5 mg b.d.†	180±7.0	7.80±0.02
Meprobamate	10 mg b.d.	116±8.2	2.82±0.02
Phenylbutazone	5 mg b.d.	209±5.0	3.96±0.02
7,12 Dimethylbenzanthracene	15 mg ‡	165±6.4	5.53±0.02
3-Methylcholanthrene	10 mg ‡	220±6.7	5.39±0.02

* Each value given is a mean of 8 experiments ± S.E.M.; tissues from 1 rat were used for 2 separate experiments. Similar values for HFC and urinary ascorbic acid were obtained when the treatment was continued for 6 days. P values for HFC between normal and treated: $p < 0.005$ for phenobarbital, chlorobutanol, chlorpromazine, phenylbutazone, 7, 12 dimethylbenzanthracene, and 3-methylcholanthrene; $p < 0.01$ for meprobamate.

† B.d. means twice daily; all other drugs were given once daily.

‡ Single administration. Chlorobutanol, 7,12 dimethylbenzanthracene, and 3-methylcholanthrene were administered as a suspension in peanut oil; the rest were administered as a suspension in water. Administration of penicillin, streptomycin, chloramphenicol, tetracycline, griseofulvin, chlorcyclizine, cortisone acetate, sulfadiazine, tolazoline, and trifluoperazine did not increase either the liver HFC or the urinary and tissue ascorbic acid.

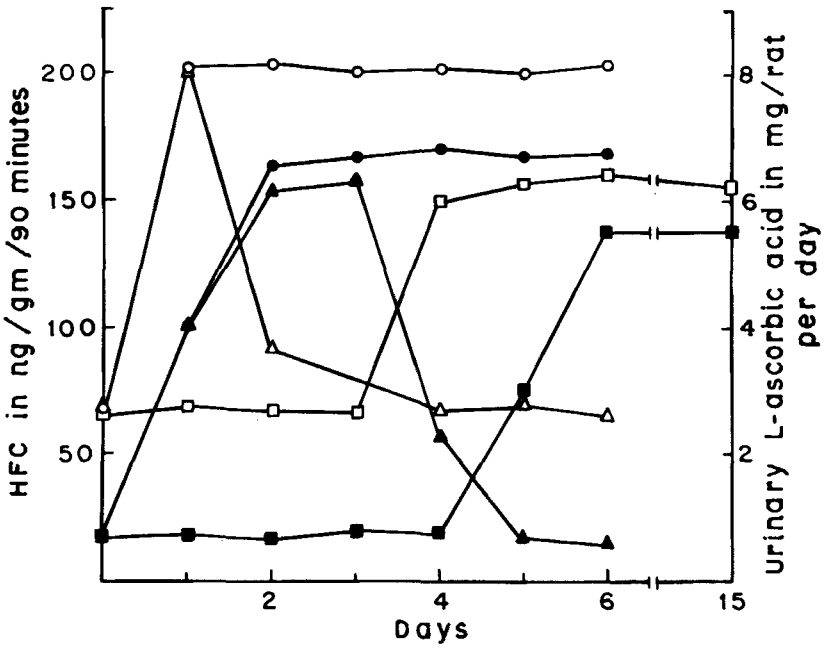


FIGURE 2. Synthesis of histamine in the rat liver and urinary excretion of ascorbic acid. The open symbols represent the value of liver HFC, and the closed symbols the urinary excretion of ascorbic acid after treatment with △——△, ▲——▲, a single dose of chlorobutanol; ○——○, ●——●, daily doses of chlorobutanol; similar results were obtained with barbital and phenylbutazone; □——□, ■——■, a single dose of 7,12-dimethylbenzanthracene. Each point in the Figure represents an average of 8 determinations. The doses given and other conditions are the same as in TABLE 1.

The relation between stimulation of HFC and ascorbic acid synthesis is also shown in FIGURE 2. Administration of a single dose of chlorobutanol, barbital, or phenylbutazone resulted in a stimulation of both HFC and urinary level of L-ascorbic acid followed by decline of the two. When administration of the drugs was continued daily, both HFC and L-ascorbic acid excretion remained elevated for the experimental period of 6 days (FIGURE 2). Further, after a single intraperitoneal injection of 7,12-dimethylbenzanthracene, none of the HFC and urinary level of ascorbic acid increased until the 3rd and 4th day, respectively, following which both HFC and ascorbic acid synthesis were enhanced and remained elevated for the experimental period of 15 days (FIGURE 2). However, how the induced formation of histamine would trigger the production of ascorbic acid in the rat liver is not clear at present.

Role of Ascorbic Acid on Detoxification of Histamine

As shown above, the enhanced synthesis of ascorbic acid appears to be related to an induced formation of histamine in the rat liver. We observed that administration of phenobarbital, chlorpromazine, and meprobamate led to stimulation of ascorbic acid synthesis accompanied by a concomitant increase (4–6 times) in urinary excretion of histamine. We considered that the production of excess ascorbic acid in response to increased histamine formation might be a natural defense mechanism for detoxicating the excess histamine. Our hypothesis was substantiated by the fact that autooxidation of ascorbic acid in the presence of histamine resulted in histamine breakdown, leading to biological inactivation of histamine, and we have indicated a function of ascorbic acid for detoxification of histamine in the body.[33]

Administration of a variety of drugs led to an increased histamine formation or release in the system, as evidenced by an enhanced histidine decarboxylase activity of gastric mucosa and increased urinary histamine level. In the rat, administration of ascorbic acid along with the drugs decreased the urinary histamine level, indicating detoxification of histamine *in vivo*.[34] In the guinea pig, histamine-producing or histamine-releasing drugs resulted in a decreased urinary ascorbic acid level, indicating greater utilization of the vitamin. A variety of stress conditions, namely administration of vaccines, toxoids, and dietary and physical stress in rats and guinea pigs led to an enhanced histamine formation or release in the system. Administration of large doses of ascorbic acid in any of the stressful situations resulted in a marked decrease in the urinary histamine level, indicating detoxification of histamine *in vivo*.[35] In the guinea pig, the utilization of ascorbic acid was significantly increased in different histamine-forming or histamine-releasing stress conditions.

Table 3 shows that blood histamine levels in guinea pigs were increased significantly after drug treatment or in different stress conditions but ascorbic acid administration brought down the levels to normal values. Figure 3 shows that ascorbic acid administration also reduces the enhanced urinary histamine content in stress condition to normal levels. The optimum dosage of ascorbic acid needed either in the drug-treated condition or in various nonspecific stress conditions was 5 mg per 100 g body weight of the guinea pig per day (Figure 4). This dose was approximately 5 times the normal need of the guinea pigs.

Scurvy and Histamine Metabolism

As shown above, ascorbic acid has a role in detoxifying histamine in different stress conditions. We considered that if the effect of ascorbic acid in detoxifying histamine was a physiological function of the vitamin, then in ascorbic acid deficiency, ascorbic acid would not be available for histamine breakdown and, as a result, the histamine levels in the blood, other tissues, and urine would increase. Figure 5 shows that in guinea pigs fed ascorbic acid free diet, as the blood ascorbic acid content began to fall, the blood level of histamine started to rise steadily, reaching a maximum long before the onset of scurvy.

TABLE 3

EFFECT OF ASCORBIC ACID ON BLOOD HISTAMINE LEVELS IN GUINEA PIGS UNDER STRESS CONDITIONS

Treatment	Blood Histamine Level (ng/ml) * Without Ascorbic Acid	With Ascorbic Acid (5 mg/100 g/Day)
None	75±5	70±2
Drugs, b.d.		
Penicillin, streptomycin	120±2	75±5
Chloramphenicol	110±2	76±4
Tetracycline	102±2	78±2
Vaccines and Toxoids, single dose		
Triple antigen	118±2	78±2
Tetanus, TABC	110±2	75±5
Cholera	105±2	76±4
Physical stress		
Heat (39±1° C)	160±5	75±5
Cold (6±1° C)	135±5	76±4
Pregnancy (50–55 days)	120±5	75±5

* 48 hours after treatment.

Similar pictures were observed in urinary excretion (FIGURE 6) and tissue histamine levels (FIGURE 7). The histamine levels did not rise if ascorbic acid (1 mg per 100 g body weight per day) was given along with the scorbutic diet. Moreover, the increased blood, urinary, and tissue histamine of guinea pigs could be brought back to normal levels by administration of a single dose of

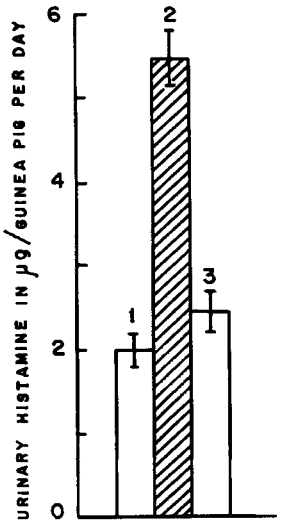


FIGURE 3. Effect of single administration of ascorbic acid (5 mg/100 g body weight) on urinary histamine level of guinea pigs under stress conditions. 1, control; 2, under stress; 3, stress condition but after administration of ascorbic acid. The stress conditions used were dietary or physical, such as cold (3–4° C), heat (39–40° C), and pregnancy (50–55 days). The vertical bars represent S.E.M.

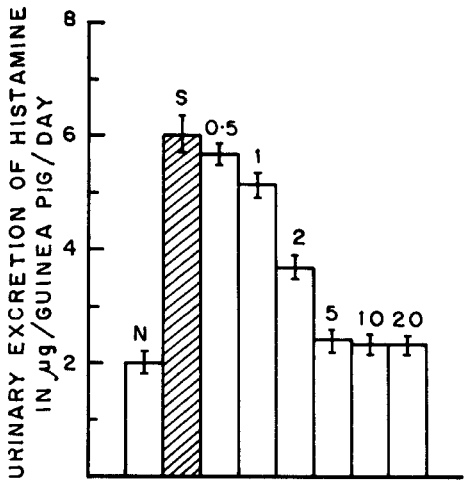


FIGURE 4. Effect of varying doses of ascorbic acid on urinary excretion of histamine under stress conditions. The stress conditions were as in FIGURE 3. N, normal; S, stress condition. The numbers over the bars represent mg ascorbic acid administered per 100 g guinea pig.

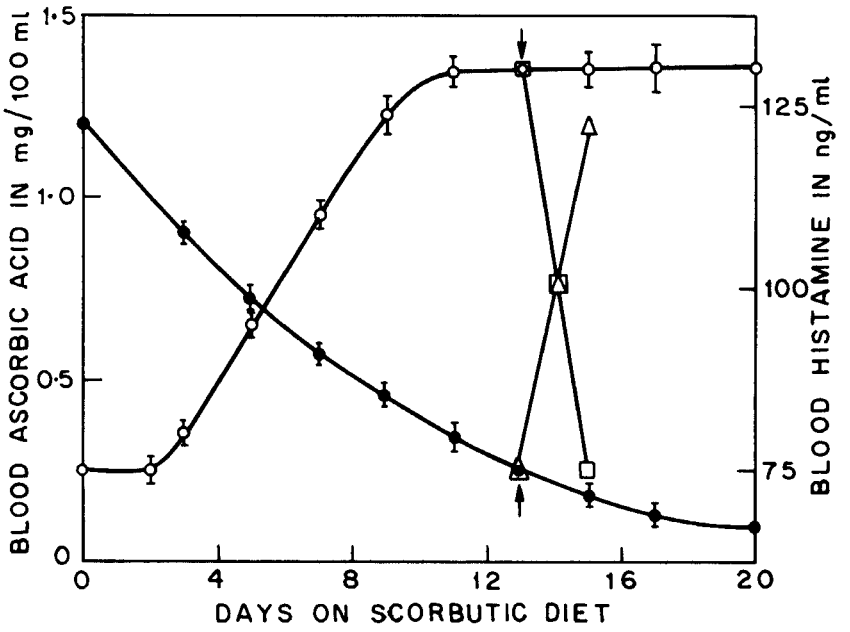


FIGURE 5. Ascorbic acid and histamine levels in blood from guinea pigs fed ascorbic-acid-free diet; ●——●, ascorbic acid; ○——○, histamine. Arrows indicate administration of a single dose of 5 mg ascorbic acid per 100 g guinea pig; △——△ and □——□ denote subsequent ascorbic acid and histamine levels. (From Subramanian *et al.*[17] By permission of the *Journal of Physiology* (London).)

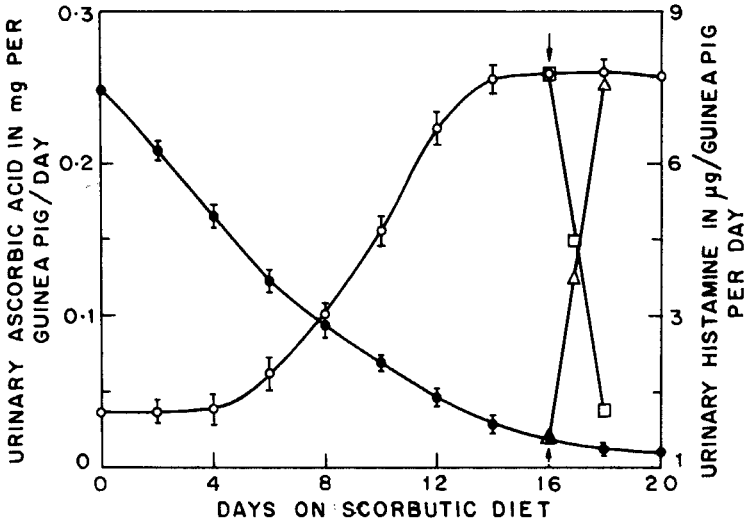


FIGURE 6. Ascorbic acid and histamine levels in urine from guinea pigs fed ascorbic-acid-free diet. Symbols as in FIGURE 5. (From Subramanian *et al.*[47]. By permission of the *Journal of Physiology* (London).)

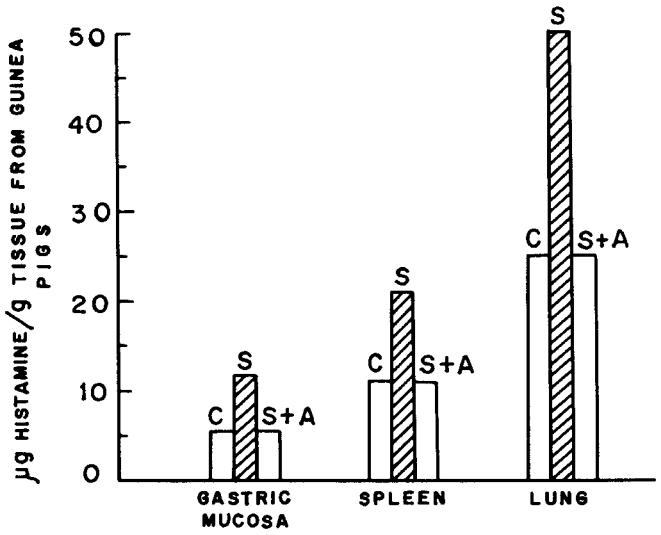
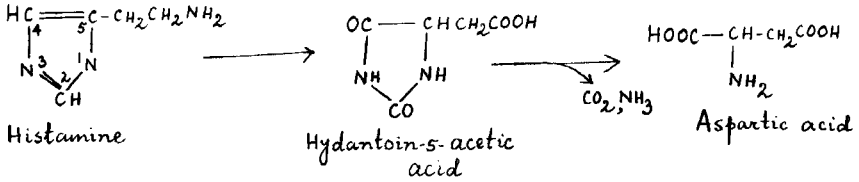


FIGURE 7. Effect of single administration of ascorbic acid on tissue histamine contents of guinea pigs fed ascorbic-acid-free diet for 16 days. The values were obtained after killing of the animals, on the 17th day. C, control; S, scorbutic diet; S+A, scorbutic diet after administration of ascorbic acid.

5 mg ascorbic acid per 100 g body weight (FIGURES 5–7). Since histamine has a strong vasodilating action on capillaries, a high histamine level would lead to hyperemia and increased capillary permeability. This would indicate that the characteristic scurvy symptom of capillary degeneration might be associated with the high histamine level in the early scorbutic condition.

MECHANISM OF ASCORBIC-ACID-MEDIATED HISTAMINE BREAKDOWN

As mentioned elsewhere,[33] neither ascorbic acid nor dehydroascorbic acid and H_2O_2, the products of oxidation of ascorbic acid, are able to break down histamine. Histamine is broken down only when it is added to a system in which ascorbic acid is allowed to undergo oxidation in presence of a catalyst such as Cu^{+2} or tissue homogenates. In a model system of ascorbic acid (5 m mole), Cu^{+2} (0.05 m mole as $CuSO_4$, $5H_2O$) and histamine (1 m mole), in a total volume of 12.5 ml 0.05 M sodium phosphate buffer, pH 7.2, incubated at 37° for 4 hours, the histamine is completely broken down to aspartic acid. Hydantoin acetic acid has been identified as an intermediate in this conversion (SCHEME 1).



SCHEME 1

The various intermediates identified at different intervals of incubation were as follows: 0 hour, histamine; 2 hours, histamine and hydantoin-5-acetic acid; 4 hours, aspartic acid. Aspartic acid was identified even after 24 hours of incubation, indicating that aspartic acid was the end product of incubation.

In the conversion of histamine to hydantoin acetic acid, the probable intermediate would be 2,4-dihydroxy-imidazole acetic acid. Apparently, this is another example of ascorbic-acid-mediated hydroxylation of heterocyclic compound. Norman and Radda [35] showed that ascorbic-acid-mediated hydroxylation could not be effected by Fenton reagent, H_2O_2, or hydroxyl radical. Involvement of perhydroxyl radical was suggested earlier by C. G. King [36] and recently by Green *et al.*[37] Presumably, histamine would first be converted to 2,4-dihydroperoxide derivation of imidazole acetic acid followed by its conversion to 2,4-dihydroxy-imidazole acetic acid, and isomerization to hydantoin-5-acetic acid.

Identification of Aspartic Acid

Aspartic acid was identified by paper chromatography (Rf. 0.23, using butanol:acetic acid:water, 8:2:2; Rf. 0.20, using phenol:water, 75:25); thin-layer chromatography using silica gel G (Rf. 0.55 using 96% ethanol:water, 7:3; Rf. 0.33 using *n*-propanol:water, 7:3; Rf. 0.21 using *n*-butanol:acetic acid:water, 8:2:2); thin-layer chromatography of the DNP derivative (Rf. 0.12 using chloroform:benzyl alcohol:acetic acid, 70:30:3; Rf. 0.07 using

chloroform:tertiary amyl alcohol:acetic acid, 70:30:3). Aspartic acid was also identified by using the glutamate-oxaloacetate transaminase reaction[39] (Rf. of glutamic acid was 0.63, with use of 96% ethanol:water, 70:30 and 0.35, with *n*-propanol:water, 70:30).

Quantitative Estimation of Aspartic Acid Produced from Histamine

With use of the model system of ascorbic acid, Cu^{+2}, and histamine, as mentioned earlier, aspartic acid was separated by band TLC on silica gel G (solvent mixture butanol:acetic acid:water, 8:2:2) and estimated quantitatively by the minhydrin color reaction. The conversion was 30% of the histamine incubated.

Identification of Hydantoin-5-Acetic Acid

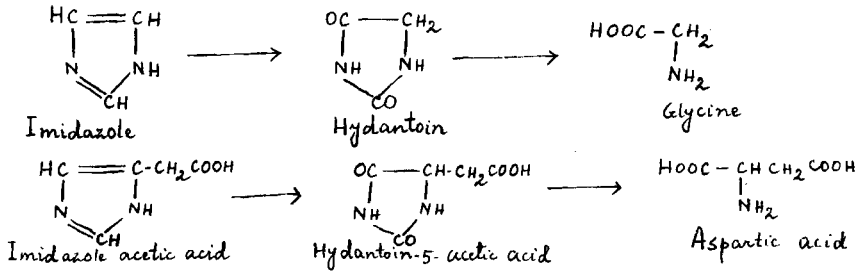
Hydantoin-5-acetic acid was identified by paper chromatography using mercuric diphenylcarbazone spray reagent of the following composition: solution A, 2% ethanolic mercuric chloride; solution B, 0.2% ethanolic diphenylcarbazone; equal volumes of solution A and B were mixed before use (Rf. 0.52 using butanol:acetic acid:water, 60:15:25; Rf. 0.71 using methanol: pyridine:water, 80:4:20); by thin-layer chromatography over silica gel G (Rf. 0.69 using butanol:acetic acid:water, 60:15:25; Rf. 0.71 using methanol: pyridine:water, 80:4:20). Hydantoin-5-acetic acid was also identified by IR spectroscopy (Figure 8) as well as by its conversion to aspartic acid in presence of ascorbic acid and Cu^{+2}, as described below.

Conversion of Hydantoin-5-Acetic Acid to Aspartic Acid

In the model system of ascorbic acid, Cu^{+2}, and hydantoin acetic acid in place of histamine as noted earlier, aspartic acid was identified as the end product after 2 hours' incubation at 37° C; 70% of the hydantoin-5-acetic acid was converted to aspartic acid.

Ascorbic-Acid-Mediated Breakdown of Imidazole and Imidazole Acetic Acid

As shown before, the imidazole nucleus of histamine is broken down in the model system using Cu^{+2} and ascorbic acid. This is further proved by using imidazole and imidazole acetic acid in place of histamine, where the products were identified as hydantoin and glycine and hydantoin acetic acid, and aspartic acid, respectively (Scheme 2).



Scheme 2

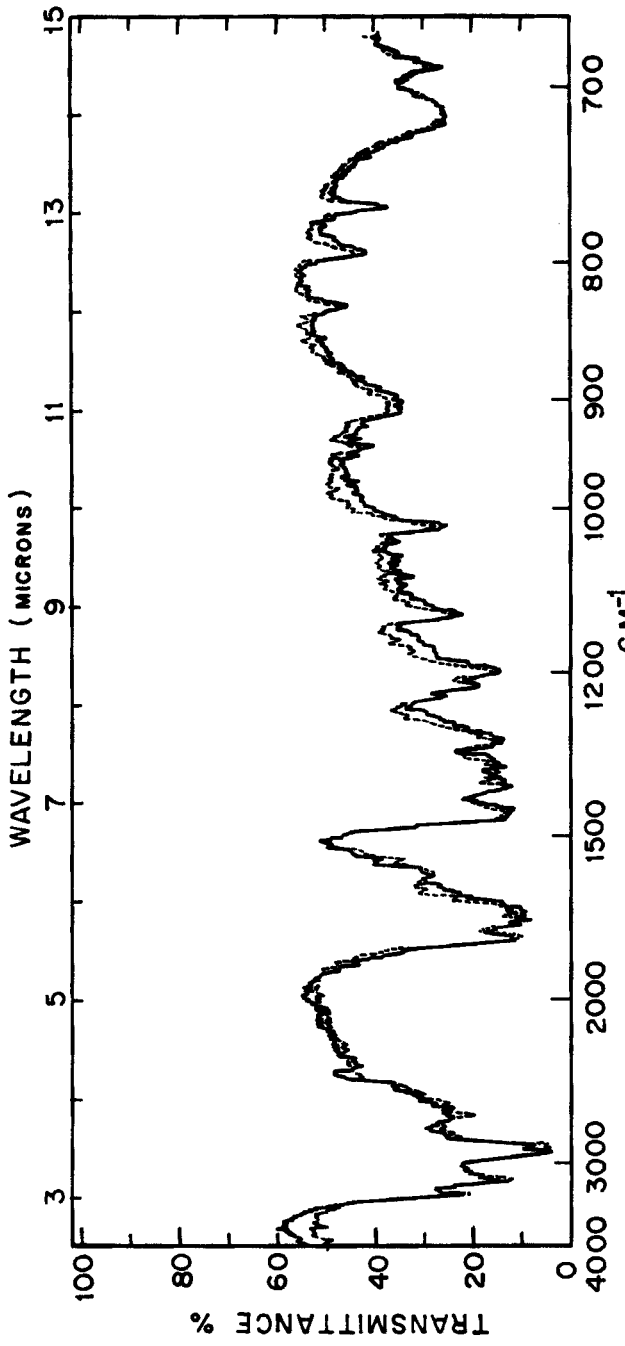


FIGURE 8. Qualitative IR spectrum of hydantoin-5-acetic acid. Solid line is that of authentic sample and dotted line is the product of incubation of histamine with ascorbic acid and copper, as noted in the text. The measurement made on a Perkin-Elmer model 137 spectrophotometer. Samples were mulled in Nujol® and run immediately.

Identification of ammonia. During ascorbic-acid-mediated breakdown of histamine, imidazole, and imidazole acetic acid, ammonia was evolved. This was identified by hanging a filter paper soaked in Nessler's reagent and dripping alkali into the reaction mixture at the end of incubation.

Experiment With ^{14}C-Histamine

Ring-2-^{14}C-histamine was separated from ring-2-^{14}C-histidine (Amersham, England) by band chromatography on cellulose powder (Sigmacell-[38]) using a solvent mixture of isopropanol, 25% NH_4OH and water (100:5:10). The ring-2-^{14}C histamine was incubated using the model system of ascorbic acid, Cu^{+2}, and cold histamine, and the $^{14}CO_2$ produced was absorbed in hyamine hydroxide [40] placed in the central well of a Warburg flask after tipping H_2SO_4 at the end of incubation. The initial count of histamine incubated was 14,775, the count of $^{14}CO_2$ in hyamine hydroxide taken in a liquid scintillation counter was 2955 and that without ascorbic acid was 558. This would confirm that ring-2-carbon of imidazole nucleus was converted to CO_2.

Effects of Large Doses of Ascorbic Acid in Guinea Pigs and Human Beings

In a previous paper [41] we indicated that large doses of ascorbic acid (AA) were toxic to guinea pigs fed high cereal diets.† At a daily dose of 60 mg of AA/100 g of body weight/day, all the 114 guinea pigs died within 16 days, and at a dose of 30 mg AA, 54 guinea pigs died within 25 days.[41] Studying the cause of toxicity of large doses of AA, we observed that under the dietary condition dehydroascorbic acid (DHAA) increased markedly in blood, urine, and liver of the guinea pigs (Table 4). Patterson [42, 43] observed that injection

† Composition in g per 100 g: wheat flour 78, cane sugar 10, peanut oil 5, shark liver oil 2, USP XVII salt mixture 4, AOAC vitamin mixture 1.

Table 4

Effect of a Large Dose of Ascorbic Acid (AA), 100 mg/100 g Body Weight/Day, on AA and Dehydroascorbic Acid (DHAA) Levels in Guinea Pigs Fed a Wheat Diet *

	Ascorbic Acid Days		Dehydroascorbic Acid Days	
	Initial	10th	Initial	10th
Blood (mg %)	0.80±0.1	1.1 ±0.1	0.02±0.01	1.2 ±0.05
Urine (mg/24 hour)	0.22±0.02	4.0 ±0.20	0.03±0.01	2.6 ±0.20
Liver (μg/g)	0.15±0.02	0.33±0.02	0.02±0.01	0.34±0.02

* Results are given from 12 guinea pigs, male 220±10 g body weight. AA was estimated by dye litration and total AA by the method of Bessey.[40] The values for DHAA were obtained by subtracting AA from total AA. DHAA was identified by thin-layer chromatography and spectrophotometrically as described elsewhere.[21]

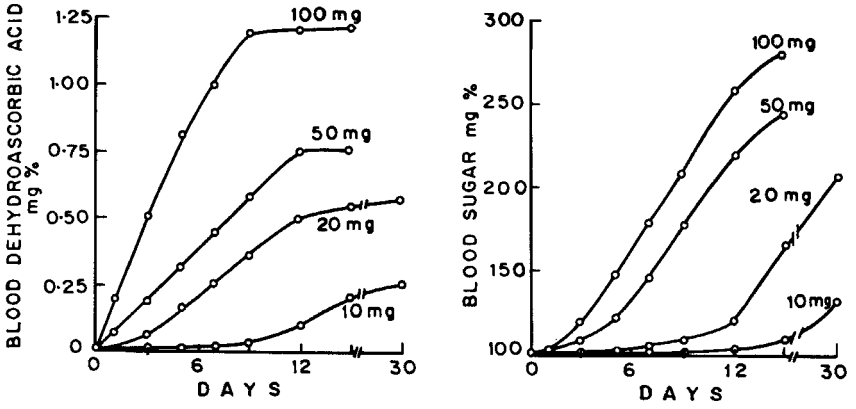


FIGURE 9. Effects of different doses of ascorbic acid on blood DHAA and sugar levels in guinea pigs fed high cereal diets. The number on each curve represents the amount of ascorbic acid administered per 100 g body weight of guinea pig per day. DHAA and sugar were estimated as mentioned in TABLES 4 and 5.

of large doses of DHAA in rats led to degranulation of β-cells of islets of Langerhans of pancreas accompanied by hyperglycemia. After feeding large doses of AA, we also observed that as the blood DHAA level increased, there was a concomitant increase in the blood sugar level (FIGURE 9). FIGURE 9 shows that after 100 mg AA per 100 g body weight/day, the blood DHAA level increased from 0.01 mg% to 1.2 mg%, with a corresponding increase of 2 hours' postprandial sugar level from 100 mg% to 284 mg% on the 15th day. When the dose of AA was reduced to 50 mg, the blood DHAA level on the 15th day was 0.75 mg%, with corresponding increase of blood sugar level to 244 mg%. When the dosages of AA was further reduced to 20 mg%, the blood DHAA level rose to 0.5 mg% on the 15th day and increased to 0.6 mg% on the 30th day, with a corresponding blood sugar value of 210 mg%. FIGURE 9 further shows that even after administration of 10 mg AA/100 g body weight/day to guinea pigs fed high cereal diet, there was a small but significant increase in both the DHAA (0.3 mg%) and the sugar level (130 mg%) on the 30th day.

The increase in blood sugar was dependent on the increase of blood DHAA level. When administration of AA was discontinued, as the blood DHAA level decreased, there was a fall of blood sugar level (FIGURE 10). On the other hand, the rise in the blood sugar did not depend on AA content of blood. When a daily dose of 100 mg AA per 100 g/body weight was administered to guinea pigs fed a fortified wheat diet,‡ the blood AA level (1.2 mg%) was similar to that obtained with high cereal diets, but the blood DHAA level did not increase and there was no increase in the blood sugar level. Also, when the aforesaid large dose of AA was administered to rats for 30 days, no increase in blood DHAA was observed, irrespective of high cereal diet or fortified wheat diet and there was no increase in the blood sugar level.

‡ Fifteen g wheat flour of high cereal diet was replaced by 15 g casein.

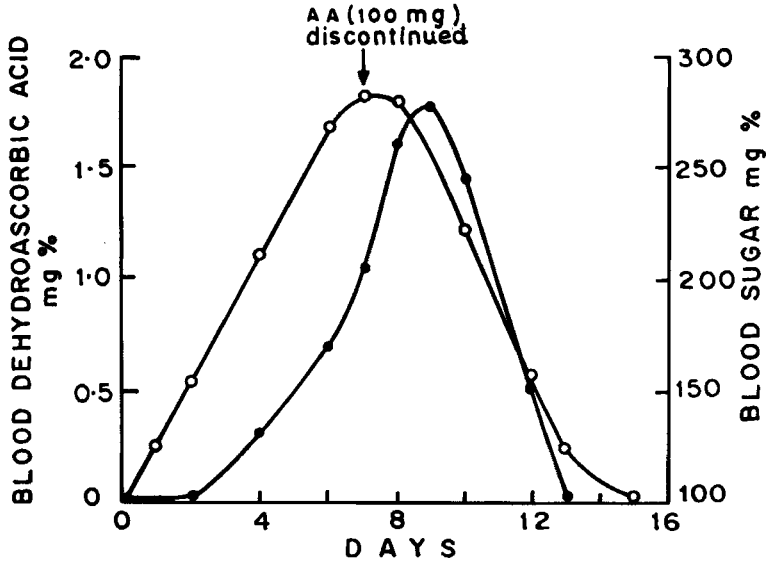


FIGURE 10. Blood DHAA and sugar levels in guinea pigs fed AA 100 mg/100 g body weight/day for 7 days. Arrow indicates discontinuation of AA administration ○——○, DHAA; ●——●, sugar. Other conditions as in FIGURE 9.

To determine the effect of nonspecific stress conditions in addition to the dietary stress, we conducted a model experiment by injecting ACTH into guinea pigs fed fortified wheat diet along with 50 mg AA/100 g body weight/day. The amount of ACTH was kept low (1 i.u./guinea pig/day), so that ACTH itself did not produce significant hyperglycemia. It was observed that ACTH injection resulted in an increased blood DHAA level followed by hyperglycemia. The blood DHAA and the 2-hour postprandial sugar levels were respectively 0.90 and 130 mg% on the 3rd day and 1.2 and 200 mg% on the 7th day. In a control group of guinea pigs that received no ACTH, neither the DHAA level increased nor was there hyperglycemia. The results indicate that intake of large doses of AA in stress conditions leads to increased blood DHAA level followed by hyperglycemia.

Feeding large doses of AA also led to high blood DHAA level in normal human volunteers fed high cereal diets (TABLE 5). After administration of 4 g AA per man per day for 15 days, all the 10 persons had marked blood DHAA increase. The blood DHAA level also increased when the dosage of AA was 2 g per man per day for 20 days. A few of the volunteers recorded higher 2 hours postprandial sugar levels. As observed with guinea pigs (FIGURE 10), in the human volunteers also, 10 days after discontinuation of AA both the blood DHAA and sugar levels came down to normal.

Accumulation of Dehydroascorbic Acid in Blood and Production of Diabetes Mellitus

The aforesaid results indicate that accumulation of DHAA in the blood leads to hyperglycemia. Since AA is an essential dietary factor of human beings,

TABLE 5

EFFECT OF LARGE DOSES OF ASCORBIC ACID (AA) ON BLOOD DEHYDROASCORBIC ACID (DHAA) LEVELS IN HUMAN VOLUNTEERS

Number * of Volunteers	Dosage of AA g/Man/Day †	Period of Intake (Days)	AA ‡ mg/100 ml 16th Day	DHAA § mg/100 ml 16th Day	Sugar ¶ mg/100 ml 16th Day
4	4	15	1.1±0.1	2.1±0.2	140±5
6	4	15	1.1±0.1	2.1±0.2	100±10
3	2	20	1.0±0.1	1.5±0.3	125±5
9	2	20	1.0±0.1	1.2±0.2	100±5

* The volunteers were males of the age group 22–40 years, without prehistory of organic disease. The diet was 80–85% cereal (wheat and rice), 8–10% legumes, some vegetables, 4–5% fish, no milk; average calorie intake was 1700.

† 500-mg Redoxon tablets (Roche, India Ltd.) 2 g b.d. or single dose, taken after meals.

‡ Initial blood AA values were in the range of 0.55–0.90 mg/100 ml.

§ Initial blood DHAA values were in the range of 0.01–0.03 mg/100 ml.

¶ Estimated 2 hours after taking 75 g glucose by the method of Nelson & Somogyi.[41] Initial postprandial sugar levels were in the range of 80–100 mg/100 ml.

we considered that the cause of diabetes mellitus in human beings might be some metabolic disorder leading to accumulation of DHAA in the blood. TABLE 6 shows that, in contrast to normal individuals, all the established diabetic patients examined had markedly high blood DHAA levels. The high blood DHAA level was irrespective of age, sex, family history, or duration of the disease detected. The patients mentioned in TABLE 6 had history of detection of diabetes from 1 month to 14 years. The 4 juvenile diabetic patients also recorded high DHAA level. In the guinea pig, there was a correlation between the blood DHAA level and the sugar level. However, such a correlation was not apparent in diabetic patients mentioned in TABLE 6. This is because all the diabetic patients examined were under treatment either by insulin or sulfonylurea derivatives. In some of the cases, the blood sugar levels were low after treatment but the blood DHAA levels were high. In these cases, when the drug was discontinued for a considerable period (4–6 months), the hyperglycemic state reappeared.

We recorded the high persistent blood DHAA levels of the established diabetic patients at different intervals for about 18 months. In none of the cases was there a significant fall in the DHAA level irrespective of the intake of antidiabetic drugs mentioned before. The high persistent blood DHAA level appears to be specific to diabetes mellitus. We examined various patients suffering from other diseases without diabetes. Although during the period of illness there was a little rise in the blood DHAA level, yet the values came down to almost normal during convalescence. Such moderately high blood DHAA levels were also recorded in various other diseases conditions but again the values came to normal during convalescence.[44]

TABLE 6

BLOOD ASCORBIC ACID (AA), DEHYDROASCORBIC ACID (DHAA) AND SUGAR LEVELS IN NORMAL SUBJECTS, DIABETICS AND PATIENTS WITH OTHER DISEASES

Subjects	Age (Years)	Sex	AA mg/100 ml (Range)	DHAA mg/100 ml (Range)	Sugar mg/100 ml * (Range)
Normal † (30)	10–55	M	0.50–1.2	0.01–0.05	70–100
Normal † (20)	20–50	F	0.45–1.1	0.01–0.05	75–100
Nondiabetic offspring of diabetic parents ‡ (8)	22–40	M	0.55–1.1	0.03–0.08	75–100
Prediabetic offspring of diabetic parents (2)	35, 36	M	0.08, 0.90	1.3, 1.0	120, 90 §
Diabetes mellitus without complications ¶ (4)	12–18	M	0.30–0.45	1.50–2.60	225–440
(2)	15, 17	F	0.35, 0.40	1.1, 1.4	200, 220
(55)	28–66	M	0.50–0.80	1.00–2.50	180–420
(20)	28–62	F	0.45–0.70	1.10–2.30	160–350
Other diseases with diabetes					
a) Hypertension || (10)	48–65	M	0.30–0.60	0.90–1.70	155–280
b) Urinary tract infection ** (5)	37–52	F	0.35–0.70	1.30–1.80	200–300
Other diseases without diabetes					
a) Hypertension †† (10)	50–70	M	0.90–1.10	0.03–0.15	90–115
b) (i) Pneumonia (5)	30–45	M	0.40–0.70	0.20–0.40	90–110
(ii) Convalescent (5)	30–45	M	0.60–0.90	0.05–0.10	80–100
c) (i) Influenza (10)	20–40	M & F	0.50–0.80	0.10–0.20	80–100
(ii) Convalescent (10)	20–40	M & F	0.70–1.00	0.03–0.08	80–100
d) (i) Urinary tract infection ** (8)	25–45	F	0.60–0.80	0.05–0.1	80–100
(ii) Convalescent (8)	25–45	F	0.60–0.90	0.03–0.05	80–100
Physical trauma					
a) (i) Burns ‡‡ (5)	20–25	M	0.30–0.50	0.40–1.00	not done §§
(ii) Convalescent (5)	20–25	M	0.60–0.90	0.05–0.20	not done
b) (i) Crushed injury ¶¶ (6)	8–30	M	0.30–0.50	0.30–1.90	not done
(ii) Convalescent (6)	8–30	M	0.70–0.90	0.05–0.20	not done

* Two hours after feeding 75 g glucose; estimated by the method of Somogyi & Nelson.[41]

† Without prehistory of organic disease; the number in the parentheses indicates the number of individuals examined.

‡ Either father or mother diabetic.

§ The blood sugar values after 6 months were 180 and 160 mg%, respectively.

¶ With or without family history of diabetes.

|| History of hypertension for 8–12 years; Range 170/90–225/100 mm Hg.

** *B. coli.*

†† History of hypertension 1–4 years; Range as in note ||.

‡‡ 15–30% burns.

§§ The patients received glucose infusion.

¶¶ Automobile accidents and fall from 20–30 feet.

Mechanism of Accumulation of Blood Dehydroascorbic Acid in Diabetes Mellitus

We considered that accumulation of DHAA in the blood of diabetic individuals might be due to a net effect of increased rate of oxidation of AA to DHAA and decreased breakdown of DHAA. However, no significant difference was observed between the rate of AA oxidation in blood cells from nondiabetic normal subjects and that from diabetics. Nevertheless, in contrast to nondiabetic subjects, the rate of DHAA breakdown was markedly low in blood cells from diabetics (FIGURE 11). Since hydrolysis of DHAA to 2,3-diketogulonic acid is carried out by a nonspecific enzyme aldonolactonase, it would appear that the activity of the enzyme 2,3-diketogulonic acid decarboxylase is inhibited in diabetic mellitus. However, whether this inhibition is a genetic defect is yet to be seen.

Mechanism of Action of DHAA *in the Production of Hyperglycemia*

Possibly DHAA did not act as an antagonist to the circulating insulin because in human volunteers who received 2–4 g AA/day, there was marked increase in the blood DHAA level without significant increase of blood sugar level. Patterson observed a gross degranulation of β-cells by injecting large doses of DHAA.[12, 13] Histological studies of the pancreas from guinea pigs fed high cereal diets and large doses of AA (100 mg/100 g body weight/day for 16 days) indicated that with a blood DHAA level of 1.6 ± 0.2 mg%, there was a significant decrease in the intensity of purple color in the islets of

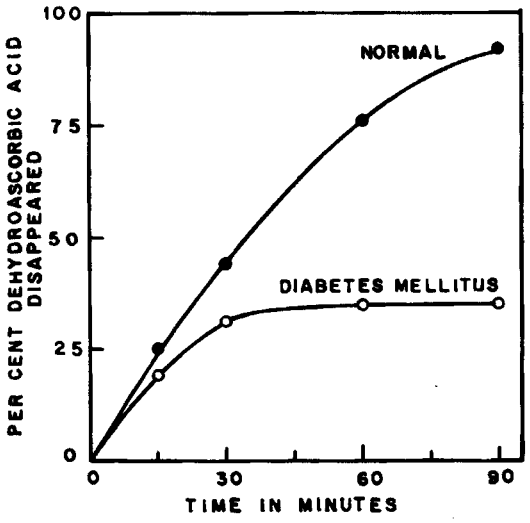


FIGURE 11. Incubation system: 200 μg DHAA incubated with 1 ml blood cell hemolysate (equivalent to 1 ml blood) in 0.1 M sodium phosphate buffer, pH 7.2 at 37°, showing rate of DHAA disappearance.

Langerhans after staining with aldehyde fuchsin with or without counterstain by ponceau. This indicates apparent degranulation of the β-cells of islets.

DHAA reacts with sulfur amino acids. Since sulfur amino acids are essential for insulin synthesis, DHAA might act by inhibiting insulin synthesis. DHAA is also known to give Strecker reaction with amino acids in general.[15] This would lead to an inhibition of protein synthesis in diabetics. Lack of protein anabolism is, in fact, one of the features of diabetes mellitus. However, the exact mechanism of action of DHAA for production of hyperglycemia is yet to be determined.

Possible Detection of the Prediabetic State

The early detection of diabetes is of tremendous importance. Easier control of the disease would be facilitated, and progression of mild to frank diabetes might be prevented. A recent population survey has shown that many apparently healthy persons, particularly from diabetic families, may have latent diabetes. We examined several nondiabetic children of diabetic parents. Two of them were physicians 34 and 36 years old. They had no clinical manifestations of diabetes. However, their blood DHAA levels were 1.3 and 1.0 mg% (AA 0.80, 0.90 mg%) and 2-hour postprandial sugar levels were 120 and 90 mg% (TABLE 6). Their diabetes became frank within 6 months, showing 2-hour postprandial sugar levels of 180 and 160, respectively. This supports the current belief that clinical manifestations of diabetes appear long after onset of the disease. According to our observation, a periodic analysis of blood DHAA content would be of value for detection of prediabetic condition.

Control of diabetes mellitus. As yet, there is apparently no cure for diabetes. Insulin and other oral antidiabetic drugs along with dietary control are employed for remission of hyperglycemia. When the drug treatment or dietary control is discontinued for a considerable period, the clinical manifestations reappear. We observed that irrespective of treatment, the blood DHAA level remained persistently high in diabetic patients. As already mentioned, when wheat diet was fortified with 15% casein, after feeding large doses of AA to guinea pigs there was neither increased DHAA formation nor hyperglycemia. It was also shown (FIGURE 10) that as the blood DHAA content decreased, the sugar level fell. These results indicate that (with exception of organic defect) diabetes mellitus could possibly be controlled by preventing formation of DHAA and dissipation of the accumulated DHAA in the blood, preferably at the early stage of the disease.

ACKNOWLEDGMENTS

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DISCUSSION

DR. B. M. TOLBERT: I have now seen the suggestion that there is a 2-keto intermediate in the synthesis of ascorbic acid proposed many times, but from a chemical point of view, I see no rationale in going from L-gulonolactone to 2-keto L-gulonolactone. I wonder why that intermediate is proposed. We know that the equilibrium between ascorbic acid and the 2-keto form of the ascorbic acid is completely toward ascorbic acid. There is obviously a very rapid reaction. So I wonder if there was some rationale for proposing that your enzyme specifically goes to the 2-keto intermediate. I would suggest that it goes directly to ascorbic acid, via biochemical or chemical processes that I am not familiar with.

DR. J. GROSS: There have been some recent reports that among a large group of guinea pigs it is possible to find some that won't become scorbutic and in fact these workers report the presence of gulonolactone oxidase in the liver. I wonder if you might comment on the possibility that there is considerable variation in the capability of synthesizing ascorbic acid, perhaps even among these species that supposedly have lost the gene.

DR. CHATTERJEE: I have not come across any such guinea pig that really could synthesize any ascorbic acid.

DR. J. J. BURNS: There is another aspect of this. Guinea pigs on a vitamin-C-deficient diet take different periods of time to develop scurvy. This can be explained by marked individual variability in rates of metabolism of ascorbic acid. Some guinea pigs can have a vitamin-C half-life much shorter than others and thus develop scurvy at a more rapid rate.

DR. C. W. M. WILSON: We reported the point about guinea pigs surviving on the scorbutic diet. I would like to ask Dr. Chatterjee whether the guinea pigs that he looked at were normal or not? What sex were they?

DR. CHATTERJEE: I suppose the guinea pigs we used were normal and we used both males and females. We did not find any synthesis either in normal conditions or in stress conditions, but it is known that the retention varies from species to species. The guinea pigs could be scorbutic in three or four weeks, whereas it takes at least three to six months for man.

DR. M. P. LAMDEN (*University of Vermont College of Medicine, Burlington, Vt.*): There are variables, like diet and other variables, that perhaps we are not immediately aware of, that may affect the variability in response of guinea pigs to a scorbutic diet. Some years ago I found out that with a certain diet there was quite a delay in scurvy formation. It turned out to be related to the use of a rancid diet. This observation seemed to be somewhat in keeping with the idea that free radicals other than ascorbic acid might possibly prevent the formation of scurvy.

DR. KITABCHI: About 14 years ago at the first conference on vitamin C, we reported on some of the biochemical properties of gulonolactone oxidase in the liver of rats. We demonstrated that this enzyme is very susceptible to lipid oxidation, particularly the unsaturated lipid moiety of the phospholipid in the microsome. Therefore, we propose that in some of the conditions where peroxidation occurs, this enzyme may be damaged. I wonder if you have studied the biochemical properties of some of these species, with particular reference to their subcellular localization as well as their susceptibility to lipid peroxidation?

DR. CHATTERJEE: We have checked on the apparently healthy animals that had really low lipid peroxide in their microsome fraction and we always used sodium pyrophosphate in a high concentration to block the possibility of any lipid peroxidation during incubation.

DR. SPRINCE: The fact that vitamin C is found primarily in warm blooded animals prompts me to ask—Is it possible that vitamin C is concerned with temperature regulation of the animal?

DR. CHATTERJEE: There are reports that it gives a beneficial effect at low temperatures.