STUDIES ON THE FORMATION OF COLLAGEN

IV. Effect of Vitamin C Deficiency on the Neutral Salt-Extractible Collagen of Skin*

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The essential role of ascorbic acid in collagen formation has been shown by numerous histological and biochemical studies (2–7). The mode of action, however, is still in doubt.

The question as to whether a deficiency of ascorbic acid prevents synthesis of collagen molecules or interferes with subsequent polymerization to fibrils was raised by Wolbach (2), who favored the latter possibility. Biochemical studies of the carrageenan granuloma by van Robertson and Schwartz (7) and of healing skin wounds in scorbutic guinea pigs by Gould and Woessner (6) led these investigators to postulate that ascorbic acid was required for the hydroxylation of proline to hydroxyproline in the polypeptide chain of a precursor of collagen. The hypothetical proline-rich, hydroxyproline-poor precursor, they suggested, is synthesized normally in the scorbutic state but not hydroxylated, and this defect accounts for the relatively large amounts of proline with little hydroxyproline found in scorbutic granulation tissues. As yet, there has not been a reported success in isolating this precursor.

Biochemical studies of collagen formation in granulation tissue (open wounds, granulomas, and implanted plastic sponges) are complicated by the variety of physiologic responses involved in healing. A method for the study of collagen formation in the intact scorbutic animal was provided by the discovery of neutral salt-extractible collagen in intact dermis (8, 9). This fraction (in tissues which are not undergoing degradation) is newly synthesized and appears to be converted to insoluble fibrils (10–12). Studies of the solubility of extracted collagen *in vitro* have indicated a possible mechanism for this process (13).

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Most of the neutral salt-extractible collagen disappears from the skin after a 2 to 3 day period of weight loss incident to caloric restriction. It reappears again 5 to 7 days after growth is resumed upon *ad libitum* feeding (10). As indicated earlier (14), the near disappearance of extractible collagen from the skin of severely scorbutic guinea pigs could not be distinguished from the similar effect of inanition on non-deficient controls. Apparently the diminished turnover of S^{35} in cartilage of severely scorbutic animals can also be duplicated by inanition alone (15).

Experiments reported in the present study were designed to avoid the complication of inanition, and are compared with the results of more conventional pair feeding. A short preliminary period of fasting eliminated the bulk of the extractible collagen from the skins of both normal and scorbutic animals; this was followed by a period of renewed feeding and consequent, nearly equal, weight gain for both, sufficient to assure reappearance of extractible collagen in the skin of the vitamin-supplemented controls.

EXPERIMENTAL

Preparation of Animals:

Group I. Pair Feeding.—Eight guinea pigs (both sexes) weighing 200 to 300 gm. were placed on a MacDonald No. 5 scorbutigenic diet (16); the four animals that served as pair-fed controls were given an oral supplement of 25 mg. of vitamin C daily. After 32 days all, including the controls, were sacrificed as described below. Two representative growth curves are shown in Fig. 1.

Another group of four animals, including two pair-fed controls, was carried for 17 days, and a third group of four animals (two controls) was studied after 10 days on the scorbutigenic diet.

Group II. Controlled Feeding.—Suckling and newly weaned animals were used in these experiments because a particularly large yield of neutral salt-extractible collagen can be obtained from the skin at this early age, and because growth rate is relatively uniform.

A. Groups of two litters of two to four guinea pigs including mothers were placed in separate cages. After 6 days on a normal diet (to eliminate animals not growing steadily at the normal rate of 6 to 10 gm. daily) the standard diet was replaced by the scorbutigenic diet for 6 more days. The young at this time were still suckling and also taking solid food. They were then separated from their mothers for 2 or 3 days and fed water only, during which time they lost from 10 to 20 gm. daily. They were returned to their cages and allowed to feed as before. Sixteen of forty test animals grew well and gained steadily for at least 5 days after recovering their lost weight. The others failed to reach their previous peak weight or levelled off too soon thereafter. Characteristic growth curves are shown for two test and two control animals from the same experiment in Fig. 2. Controls were prepared in the same manner except for an orally administered supplement of 25 gm. of ascorbic acid. Test animals were killed 8 to 9 days after restoration to an *ad libitum* but deficient diet, a total of 18 or 19 days of ascorbic acid–deficient feeding.

B. One group of six scorbutic animals, on the above described regimen, which failed to gain weight in the final stage of *ad libitum* feeding, were then given ascorbic acid daily for 2, 4, and 8 days (four animals), during which period they increased their weight steadily (Fig. 3). These animals were then studied as described.

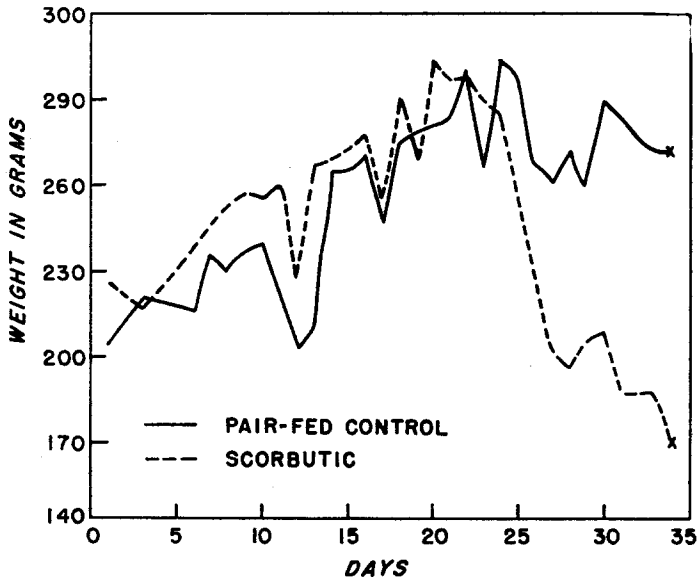

FIG. 1. Growth curves for a guinea pig carried to extremis on a scorbutigenic diet and its pair fed vitamin C–supplemented control.

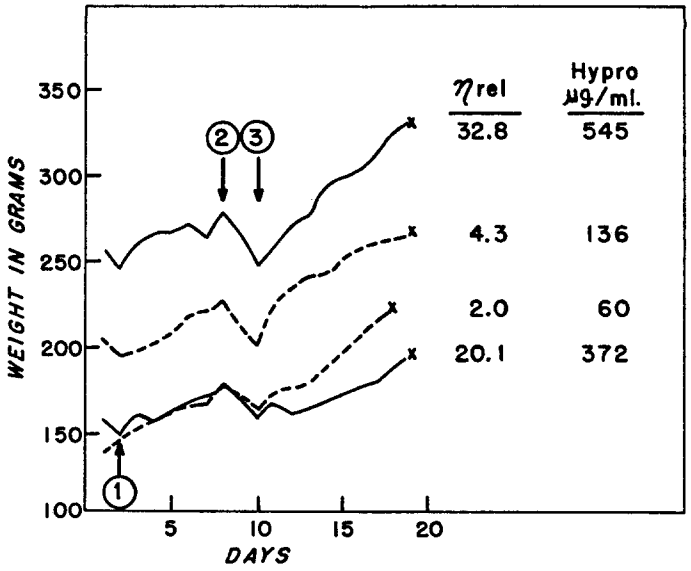

FIG. 2. Growth curves for 4 animals of a single representative experiment of group II A. Arrow 1 indicates time the animals were placed on the scorbutigenic diet. Arrow 2 indicates the beginning of the 2 day fasting period. Arrow 3 indicates the end of that period and beginning of *ad libitum* feeding. Solid lines represent the vitamin-supplemented controls. The dotted lines are for the vitamin C–deficient test animals. Relative viscosity and hydroxyproline concentrations of the extract derived from the skins of each of these animals are recorded adjacent to each curve.

Preparation of Extracts:

All animals were sacrificed by intraperitoneal injection of nembutal and exsanguination by cardiac puncture. Whole fresh blood was analyzed for ascorbic acid (through the courtesy of Dr. Charles Dutoit and the Clinical Chemistry Laboratory of the Massachusetts General Hospital by the method of Roe and Keuther (17)). Jaws were examined histologically by the method of Goldman and Gould (18) for severity of scurvy.

The skins were removed, the epidermis and subcutaneous tissues scraped off, and the dermis ground in a hand-driven meat grinder with dry ice as previously described (19). The

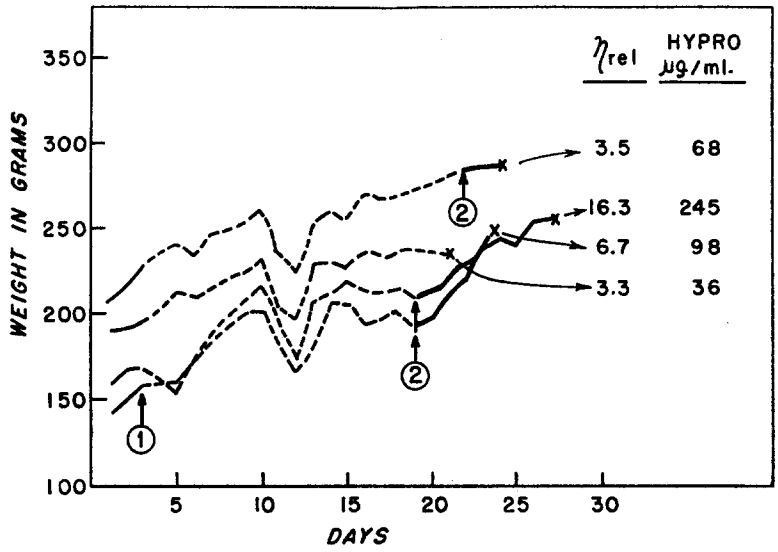

FIG. 3. Growth curves for 4 animals of group II B. Arrow 1 indicates time at which all 4 animals were placed on the scorbutigenic diet. Arrow 2 indicates the time of addition of vitamin C to the deficient diet. The solid portion of the curves represent growth during the final stage of supplementation with ascorbic acid at the end of the deficient period. The animal represented by the top curve received ascorbic acid supplement for 2 days. The animal represented in the lowest curve was supplemented for 4 days. The curve coinciding with the highest viscosity and hydroxyproline represents the animal which received the vitamin C supplement for 8 days. The remaining curve representing a non-supplemented animal corresponds with the lowest viscosity and hydroxyproline values.

coarsely ground dermis was extracted in 2 volume of cold 0.45 M NaCl at 4°C. for 16 hours on a wrist shaker. The extracts were separated from the residue by centrifugation in the Spinco model L preparative ultracentrifuge for 2 hours at 50,000 *G* then filtered through fine sintered glass with suction. In some instances the tissue residues were extracted repeatedly until hydroxyproline was no longer detectible in the extract. All manipulations were carried out at 3–5°C. Merthiolate 1:5000 was used as a bacterial suppressor.

Analyses of Extracts:

Crude extracts were analyzed for hydroxyproline (20), proline (21), glycine (22), and tyrosine (23) before dialysis and, in some cases, after dialysis against 0.41 M NaCl. Analyses

for the first three amino acids were made on hydrolysates prepared by heating 1 ml. of extract with 1 ml. of 12 N HCl in a sealed glass tube in an oil bath at 138°C. for 3 hours. Tyrosine was measured in an alkaline hydrolysate. Hexosamine was determined by the method of Boas (24).

Viscosity of all extracts was determined in an Ostwald viscometer with 60 second flow time for water at 5°C. Several extracts representative of each group of animals were examined in the analytical ultracentrifuge (Spinco model E) at 56,000 R.P.M. at 2–8°C. using a wedge cell. Samples were dialyzed against veronal citrate NaCl buffer pH 8.6 $\Gamma/2$ = 0.2 and subjected to moving boundary electrophoresis at 1°C. in the Perkin-Elmer apparatus. Specific optical rotation of purified collagen solutions was measured with a Rudolf photoelectric polarimeter at +5° and +25°C.

Fractionation of Extracts: (Group II A, four animals; Group II B, two animals.)

Fractionation of the crude extracts from each animal was accomplished by the trichloracetic acid–ethanol procedure described previously (19). In addition, however, fraction IIp was further subdivided by dissolving the wet trichloracetic acid precipitate in phosphate buffer, pH 7.8 $\Gamma/2$ = 0.4 (Fig. 6). The small insoluble residue was separated by centrifugation and the supernatant fluid dialyzed against phosphate buffer to free it of trichloracetic acid. To the clear supernatant fluid was added cold (+1°C.) absolute ethanol to a final concentration of 14 per cent. After standing at 3–5°C. for 16 hours the precipitate was removed by centrifugation and redissolved in phosphate buffer. A very small insoluble residue remained, and this was dialyzed against water and lyophilized. The phosphate solution was then examined in the analytical ultracentrifuge, in the electrophoretic apparatus (after dialysis against the veronal buffer described above), dialyzed free of salt, and lyophilized. The supernatant fluid containing the alcohol was dialyzed against veronal buffer for electrophoresis, then dialyzed free of salt, and lyophilized. The residue remaining from the early phosphate extraction of fraction IIp was suspended in water and refluxed at 100°C. for 3 hours to solubilize any collagen as gelatin, then separated into supernatant and residue both of which were dialyzed against water and lyophilized. Each fraction was dried *in vacuo* at 108°C. for 16 hours, weighed, and analyzed for hydroxyproline, proline, and glycine.

RESULTS

The scorbutic state was manifested in the test animals by the presence of less than 0.1 per cent ascorbic acid in the blood (usually not measurable), the presence of hemorrhage in the skin and periarticular tissues, ease of separation of the jaw bones from soft tissue, and by alterations in the odontoblasts as described by Goldman and Gould (18).

Group I.—As illustrated in Table I, the lowest hydroxyproline concentrations were obtained in animals on the scorbutigenic diet for 32 days; the wide variation in hydroxyproline content of extracts of the control animals is a result of differences in their growth rate. A considerable difference between normal and control extracts was found after only 17 days on the diet, but 10 days of deficiency did not produce a significant diminution of extracted hydroxyproline. There was little difference between the growth curves for this last group of normal and deficient animals.

The group of test animals allowed to feed *ad libitum* began to lose weight after 15 to 20 days and were obviously sick when sacrificed on the 32nd day. The

pair-fed controls did not lose as much weight as did the test animals (Fig. 1). Fig. 4 is a protocol illustrating the differences between skin extracts of a representative pair of test and control animals in terms of hydroxyproline, tyrosine, hexosamine, and sedimentation pattern. Proline and glycine determinations were not made in this series of experiments. Hydroxyproline values (as shown in Table I) were significantly lower in the scorbutic extracts; tyrosine was unchanged (14) and hexosamine was slightly elevated.

The ultracentrifuge pattern of the normal extract (Fig. 4) revealed a large, relatively fast-moving diffusing boundary followed by the hypersharp slow moving peak representing the collagen fraction as previously described (19).

TABLE I

Hydroxyproline Concentration in the Crude NaCl Extracts from the Scorbutic and Pair-Fed Control Animals of Group I

Days on scorbutic diet	Hypro in μg./ml.	
	Controls (pair-fed)	Scorbutic
32	78	35
	142	39
	117	38
	84	30
17	182	61
	395	65
10	340	231
	527	507

The pattern of the scorbutic extract lacked even a suggestion of the collagen boundary.

The extract of the skin from the pair-fed control animal (sedimentation pattern shown in Fig. 4 *a*) was diluted with buffer so that it contained less hydroxyproline than did the extract of the scorbutic animal and reexamined in the ultracentrifuge. A small but definite collagen boundary was still visible in the diluted normal extract (Fig. 4 *b*), indicating that either the small amount of hydroxyproline present in the scorbutic extract was not part of a normal collagen molecule or that the colorimetric analysis in this case measured another chromagen.

Group II A.—In the series of forty scorbutic animals on the regulated dietary regimen sixteen animals showed growth patterns nearly identical with those of the non-deficient controls. These animals had the usual stigmata of scurvy, including tissue hemorrhages and loosening of the attachments of muscle to the jaw bones, but they were not outwardly sick.

The analytical data obtained from the crude extracts of skins of all the normal and scorbutic animals in this series are given in Fig. 5. The cold saline extracts from scorbutic skins had low viscosity and low hydroxyproline and proline contents as compared with the equivalent extracts from normal animals. The values for "excess proline," which represent the amount of this amino acid in excess of that accounted for by the extracted collagen (assuming a percentage of proline equal to that of hydroxyproline in collagen), were also diminished in the scorbutic as compared with the normal. Electrophoretic and

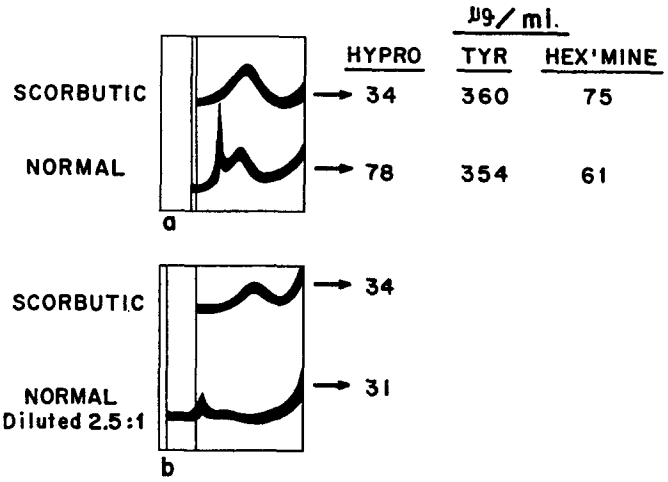

FIG. 4. (*a*) Sedimentation patterns of extracts from the skins of the two animals described in Fig. 1. The top diagram represents the scorbutic extract and the bottom that obtained from the pair-fed control. Hydroxyproline, tyrosine, and hexosamine concentrations accompany the two diagrams.

(*b*) Represents the sedimentation patterns of the same two extracts described above except that the normal extract was diluted so that its hydroxyproline content was essentially the same as that of the scorbutic extract. Note the presence of the small, sharp peak in the normal and its absence in the scorbutic.

ultracentrifuge patterns of representative normal and scorbutic extracts revealed a relatively low collagen boundary in the scorbutic extract as compared to the high spike in the normal. Electrophoretic and sedimentation patterns of skin extracts of scorbutic animals failing to gain weight during the final *ad libitum* feeding stage did not reveal any definite collagen boundary, whereas extracts from non-scorbutic animals behaving in this manner always showed a small peak characteristic of collagen.

Group II B.—The group of four scorbutic animals which received ascorbic acid for 8 days after failing to grow during the third stage of refeeding gained weight steadily on the vitamin supplement (Fig. 3). Skin extracts were very

viscous and contained a relatively high content of collagen (η rel. = 14, 16, 16, 19; hypro = 245, 252, 267, 267 μg./ml). As illustrated in Fig. 3 the viscosity and hydroxyproline values in the extract obtained from the skin of the

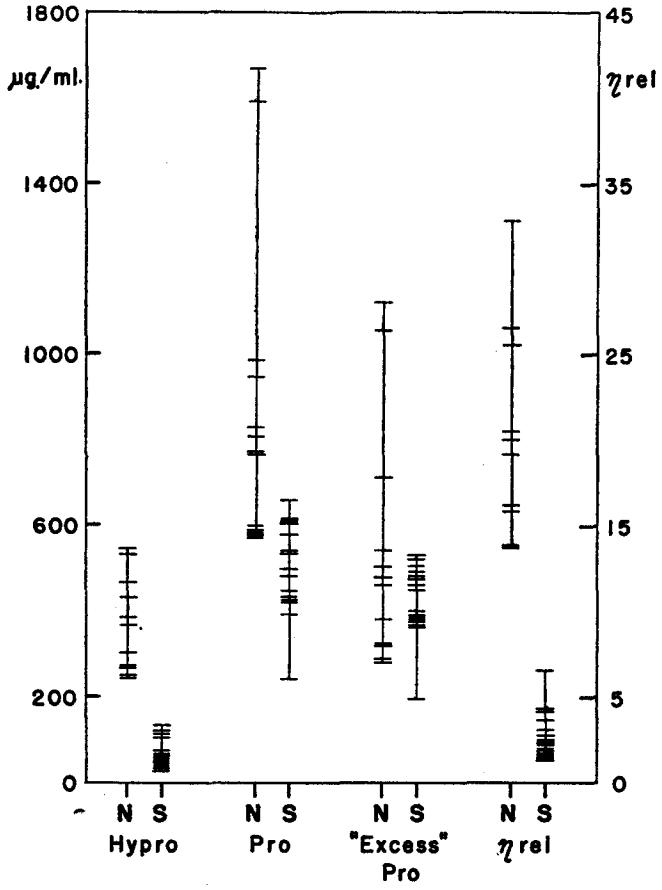

FIG. 5. Relative viscosity, hydroxyproline, proline, and calculated excess proline of all the normal (N) and scorbutic (S) extracts of group II A. Each horizontal line represents the value for a single extract.

animal treated for only 2 days were nearly as low as those for the non-treated. Four days of treatment produced a slight increase in extractible collagen.

Fractionation.—Separation of the extracts from both scorbutic and vitamin C supplemented controls into seven fractions (Fig. 6 and Table II) produced only two which contained appreciable amounts of collagen as identified by sedimentation and electrophoretic patterns, heat precipitation, hydroxyproline, proline, and glycine content.

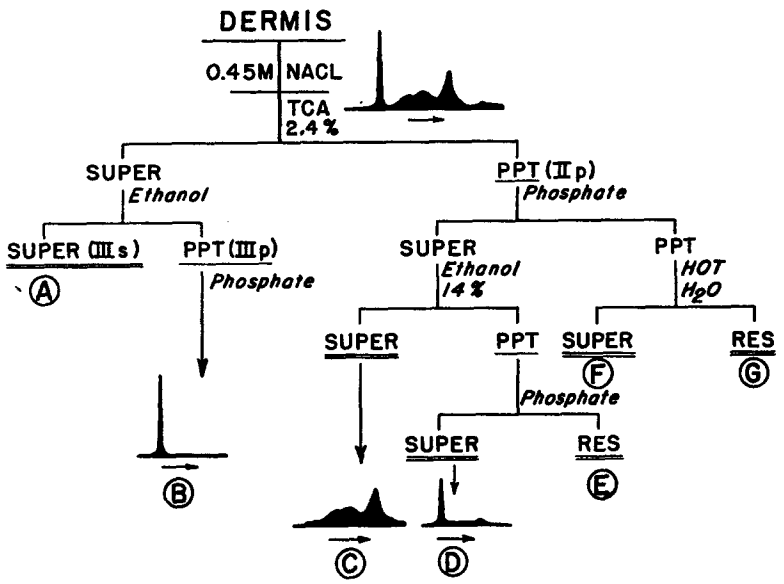

FIG. 6. Scheme of fractionation of crude NaCl extracts from normal and scorbutic animal skins. Lyophilized salt-free fractions A to G were obtained for analysis. Reproductions of the electrophoretic patterns (ascending limbs) of the original crude normal extract and fractions B, C, and D are illustrated. Details of the left hand portion of this fractionation scheme were reported in an earlier publication (19).

TABLE II

Analysis of Fractions

Analyses of Fractions A through G Obtained via the Scheme Described in Fig. 6 Isolated from Representative Extracts Obtained from the Dermis of One Normal and One Vitamin C–Deficient Animal from Group II A

Fractions	A	B	C	D	E	F	G
	Per cent of total non-dialyzable solids in extracts						
Normal	14	20	23	7	10	4	21
Scorbutic	15	6	38	4	6	3	24
	Per cent hydroxyproline in each fraction						
Normal	0.2	13	0.2	6	2	2	0.2
Scorbutic	1	10	0.2	3	0.4	0.4	0.2
	Per cent proline in each fraction						
Normal	1	11	3	6	3	3	3
Scorbutic	8	8	3	3	3	3	3
	Per cent glycine in each fraction						
Normal	1	25	3	13	7	4	3
Scorbutic	1	19	2	8	2	2	3
	Ratio of hypro:pro:gly in μg./(mg. solid/ml. extract)						
Normal	1:8:6	1:1:2	1:12:10	1:1:2	1:2:3	1:2:3	1:14:14
Scorbutic	1:7:7	1:1:2	1:13:9	1:1:2	1:7:6	1:20:18	1:16:16

Fraction B from the control extracts was an essentially pure collagen, whereas the much smaller amount of collagen in this fraction of the scorbutic extracts was usually contaminated with unidentified substances; the contents of the three amino acids were 60 to 90 per cent that of the control fractions. However, the proportions of the three amino acids relative to each other were the same for both normal and scorbutic.

Specific optical rotation of the collagen in fraction B using hydroxyproline as a measure of collagen content (assuming 13.6 per cent as that of pure collagen) ranged from $[\alpha]_{25^\circ}^{D}$ $-360°$ to $-440°$ for both normal and scorbutic.

The sedimentation diagrams obtained with fraction B from a scorbutic extract containing only 60 per cent of the normal hydroxyproline, proline, and glycine content revealed only the usual single hypersharp boundary. Fraction B from scorbutic extracts when dissolved in cold phosphate buffer pH 7.6 $\Gamma/2$ = 0.4 formed a rigid opaque gel when warmed to 37°C. in a manner indistinguishable from that of the normal.

Fraction D (Table II) contained a considerably smaller amount of collagen but here again hydroxyproline, proline, and glycine were found in the usual relative proportions. The collagen present in fraction D, amounting to 20 to 25 per cent of the total collagen in the extract, was carried down in the trichloracetic acid precipitate, probably by mechanical entrapment and by combination at the acid pH with relatively small amounts of acidic high polymers to form an acid-insoluble complex. It was recovered by dissolving the trichloracetic acid precipitate in cold phosphate buffer and precipitating out the collagen with ethanol. This precipitate, redissolved, was called fraction D.

Fraction F of the scorbutic extracts, which accounted for only 3 to 4 per cent of the total extracted solid, contained ten times as much proline and six times as much glycine with respect to hydroxyproline as did the normal. Fraction E revealed changes in the same direction but much less marked. However, it is readily apparent on inspection of Table II that if these three amino acids were part of a single molecule in these fractions, this molecular species would account for a very small proportion of the total solid extracted. None of the other fractions obtained from the scorbutic extracts revealed a significant variation in proportions of the three amino acids from that found in the normal.

DISCUSSION

It would appear that in the intact cutaneous connective tissue of the severely scorbutic animal there is a complete loss of collagen extractible by cold neutral salt solutions. No collagen boundary was demonstrable in the ultracentrifuge and electrophoretic patterns of the skin extracts. The small amount of hydroxyproline detected on analysis may have been present in a collagen fragment, or as free hydroxyproline, or the color might have been due to some unidentified chromagen. Extracts prepared from a few non-deficient animals that had lost

as much as one-third of their body weight as a result of underfeeding with a normal ration of ascorbic acid always contained a small amount of identifiable collagen (10). These observations suggest that a low level of collagen synthesis persists even in the face of extreme inanition provided that ascorbic acid is available, whereas in the scorbutic animal either synthesis stops or a degradative process destroys whatever small amount of collagen is produced.

The total collagen removed by exhaustive repeated extraction parallels the differences between single extracts of the normal and scorbutic skins (14).

It is of some interest that pair-fed controls never lost as much weight as did the experimental animals; thus the pair-feeding procedure does not provide satisfactory controls for this type of experimentation. It is also interesting to note that relatively normal amounts of neutral salt-extractible collagen can be obtained from the skin of an animal after 10 days on an ascorbic acid–deficient diet if the animal is growing at a normal rate. This could mean that either collagen synthesis continues as usual during this period or that the neutral salt–extractible collagen produced prior to deficiency is for some unknown reason not rendered insoluble during the early days of deficiency.

It is evident that active growth, which normally stimulates the production of neutral salt-extractible collagen, fails to do so in the scorbutic animal. The small amount of extractible collagen obtained under these conditions, when isolated, has the usual characteristics of the normal protein, although it appeared to be contaminated with variable amounts of another, as yet unidentified, substance.

Analysis of the crude skin extracts did not reveal a greater excess of proline in the scorbutic, as had been found by Gould and Woessner (6) on analysis of granulation tissues from skin wounds. It should be noted, however, that the present studies showed that a considerable amount of non-collagenous proline (about one-fourth of which was dialyzable) was present in the extracts of both normal and scorbutic animal skins. Little or no dialyzable hydroxyproline was found.

Separation of the scorbutic extract into seven separate fractions did not reveal the presence of a constituent which contained an unusual excess of glycine and proline as compared to hydroxyproline in amounts to suggest that a soluble precursor of this nature is produced in the scorbutic state which only awaits hydroxylation of proline to become evident as newly formed collagen.

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SUMMARY

The skin of severely scorbutic guinea pigs which were losing weight contained no detectible neutral salt-extractible collagen.

Conditions of growth (weight gain) which actively induced the formation of neutral salt–extractible collagen in the skin of normal guinea pigs failed to do so in the animal with ascorbic acid deficiency.

No excess of non-collagenous proline was found in neutral salt extracts of scorbutic skin as compared with normal.

Fractionation of these extracts failed to reveal the presence of significant amounts of a soluble component containing unusual proportions of glycine and proline relative to hydroxyproline.

It is concluded that deficiency of ascorbic acid either interferes with the synthesis of new collagen in intact skin or causes its destruction and removal as rapidly as it is produced.

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