ASCORBIC ACID-INDEPENDENT AND ASCORBIC ACID-DEPENDENT COLLAGEN-FORMING MECHANISMS*

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That ascorbic acid plays a definite role in collagen biosynthesis has been amply demonstrated by the fact that after relatively short withdrawal of ascorbic acid from the diets of ascorbic acid-dependent animals, collagen formation in healing wounds and in model systems, as in implanted polyvinyl sponges and in carrageenan granulomas, is markedly impaired. It has also been demonstrated[1,2] that ascorbic acid is essential to prevent the almost complete resorption of newly formed collagen as studied by the polyvinyl-sponge technique. What, among other things, is not as clear is whether all collagen biosynthesis and maintenance is ascorbic acid-dependent.

In a previous investigation, Woessner and Gould[3] found, in a tissue culture study of collagen formation by chick embryo fibroblasts, that collagen was synthesized even in the absence of ascorbic acid. This appeared to be strikingly opposed to the generally accepted concepts and to the subsequent finding by Gould[4] that indicated a local and direct action for ascorbic acid in collagen biosynthesis. This led to the suggestion that more than one mechanism for collagen synthesis may exist: one such as that involved in normal body collagen formation and another that predominates in tissue repair. The former could be visualized as a slow and stable mechanism relatively independent of ascorbic acid, while the latter would be rapid, perhaps less stable, and ascorbic acid-dependent.

Several other observations point to such a possibility. In earlier work on collagen formation in regenerating skin[5] and in sponge implants,[4] it was always found that in the absence of ascorbic acid, collagen formation was markedly impaired but never completely inhibited. It usually was about 15 to 20 per cent of normal. Histological examination always showed a fine collagenous capsule around sponge implants in deficient animals and a dense capsule around sponges in normal animals. Furthermore, while collagen formation in wounds and sponges essentially ceases after six to seven days' withdrawal of ascorbic acid from the diet, young guinea pigs continue to grow at what appears to be a normal rate for a considerable time beyond this depletion period.

It was this last observation that suggested a possible means to test the hypothesis that more than one mechanism for collagen biosynthesis exists and to determine whether there might be both ascorbic acid-independent and dependent mechanisms.

The experiments involved, first, a study of total collagen synthesis in young guinea pigs placed on the scorbutigenic diet from birth compared with collagen synthesis in litter-mates that were given supplements of ascorbic acid. Then, after depletion periods, skin wounds were made or polyvinyl sponges implanted,

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and collagen formation in these systems could be compared in animals on deficient and supplemented diets to determine whether similar or different mechanisms from those operating in growth were involved. The data to be presented point quite definitely to the possibility of more than a single mechanism and suggest that both ascorbic acid-independent as well as ascorbic acid-dependent mechanisms exist.

Methods

Animals. Newborn animals, weighing from about 60 to 80 gm. were used in most cases. The young animals were placed on a scorbutigenic diet immediately. Litters were divided so that experimental animals were compared with litter-mates whenever possible. Animals were fed by hand at the outset, but they soon fed themselves. Animals that refused the diet or gained no weight were discarded. Animals rarely showed any signs of apparent scurvy before the 16th day and rarely died before the 30th day. Normal controls were fed the scorbutigenic diet supplemented daily with 10 mg. of L-ascorbic acid given orally. In most instances animals were maintained on the scorbutigenic diet for 6 days before wounding[5] or before implanting the polyvinyl sponges.[4] Wounds were usually made by excising ¾-in. circles of skin bilaterally and, generally, four 10-mg. sponges were implanted, two on each side of the animal.

Collection of tissue. For total collagen formation, the collagen content of the skin and that of the carcass were determined separately. The hair was removed by plucking as completely as possible, and the entire skin was removed, weighed, frozen in dry ice, and ground in an electric meat grinder. The ground skin was then extracted repeatedly in the cold with 0.45 *M* NaCl to remove salt-soluble collagen. The carcass, minus the head, a long bone, and a small piece of costochondral junction needed for histological examination, was weighed, minced, and then blended in cold 0.45 *M* NaCl and repeatedly extracted to remove salt-soluble collagen.

Regenerating tissue of skin wounds was isolated by careful dissection, and sponges were removed by forceps through a small incision, using care not to include preformed collagenous tissue or the capsule that usually surrounds the sponge.

When *total collagen* hydroxyproline was to be determined, the tissue was dried in acetone for 24 hours, minced finely, and re-extracted with acetone for another 24 to 48 hours, then defatted with ethyl ether, and dried at 108° C. *in vacuo*.

Soluble collagen. After each 24-hour shaking period in the cold, the salt extract was collected by centrifugation according to the method described by Gross,[6] and the tissue re-extracted in the same way five to six times. The combined clear extracts were then dialyzed in the cold against large volumes of distilled or tap water to remove salt and low-molecular-weight compounds. The dialyzed material composed of precipitate and suspending fluid was homogenized, and an aliquot was taken for collagen analysis.

Insoluble collagen. The residue after the extraction with 0.45 *M* NaCl was suspended in water and treated as follows:

1. *Isolation of collagen.* The collagen in the acetone-treated sponge or

tissue, in the dialyzed soluble extract, or in the residue containing the insoluble collagen was converted to gelatin by autoclaving twice for 3 hours each time at 25 psi steam pressure. The extracts were combined and dried in a steam bath in a current of air.

2. *Hydrolysis of the gelatin extract.* The dried autoclave-extractable material was hydrolyzed in 6 *N* HCl in a sealed tube by heating at 150° C. for 3 hours. The hydrolyzate was adjusted to neutrality either by adding the theoretical amount of NaOH or by removing the HCl by repeated evaporation.

Hydroxyproline determination. In view of the essentially unique occurrence of hydroxyproline in collagen, it can be used as a measure of the collagen content. The hydroxyproline content multiplied by 7.46 may be used as a measure of the apparent collagen content. The method employed was that of Neuman and Logan[7] as modified by Martin and Axelrod.[8]

Experimental

Growth studies. Several litters of newborn guinea pigs were divided into groups so that litter-mates could be maintained on both ascorbic acid-supplemented and ascorbic acid-free diets for varying periods of time. Growth was determined by weighing the animals each morning before feeding. Particular attention was paid through the first 14 days that covered the period involved in subsequent wounding and implantation experiments. A certain number of animals, on both the supplemented and scorbutigenic diets, that refused to eat were discarded.

It is obvious from FIGURE 1 that during the first two weeks after birth the growth rate of guinea pigs, on the diet provided, was essentially independent of added ascorbic acid. Beyond 15 or 16 days it was found that the animals on the unsupplemented diet no longer gained weight as did those receiving ascorbic acid, but they continued to appear in good health for as long as 25 days. Some animals on the scorbutigenic diet subsequently lost considerable weight, but they were generally among those that during the earlier period had gained the most.

Total collagen of animals on scorbutigenic and ascorbic acid-supplemented diets. A large number of animals maintained on the scorbutigenic diet and others that received a daily supplement of ascorbic acid for periods of 15 days or more and that had attained weights of 150 gm. or more were analyzed to determine both the total soluble and insoluble collagen content of the animal. A series of newborn animals were analyzed directly after birth to indicate the base level. Another series was analyzed after six days on the scorbutigenic diet on the assumption that a certain amount of synthesis might occur as a result of ascorbic acid carried over from the mother. A six-day period has been found adequate to deplete the tissues enough to prevent collagen synthesis in ascorbic acid-dependent systems.

An examination of TABLE 1 indicates quite clearly that even in the absence of ascorbic acid young guinea pigs during growth were able to synthesize very substantial amounts of collagen. Only a small fraction was formed during the first six days after birth when there may still have been some residual ascorbic acid present. The rate of synthesis during this period appears to be no greater than during the later period. The total collagen synthesis appears to be di-

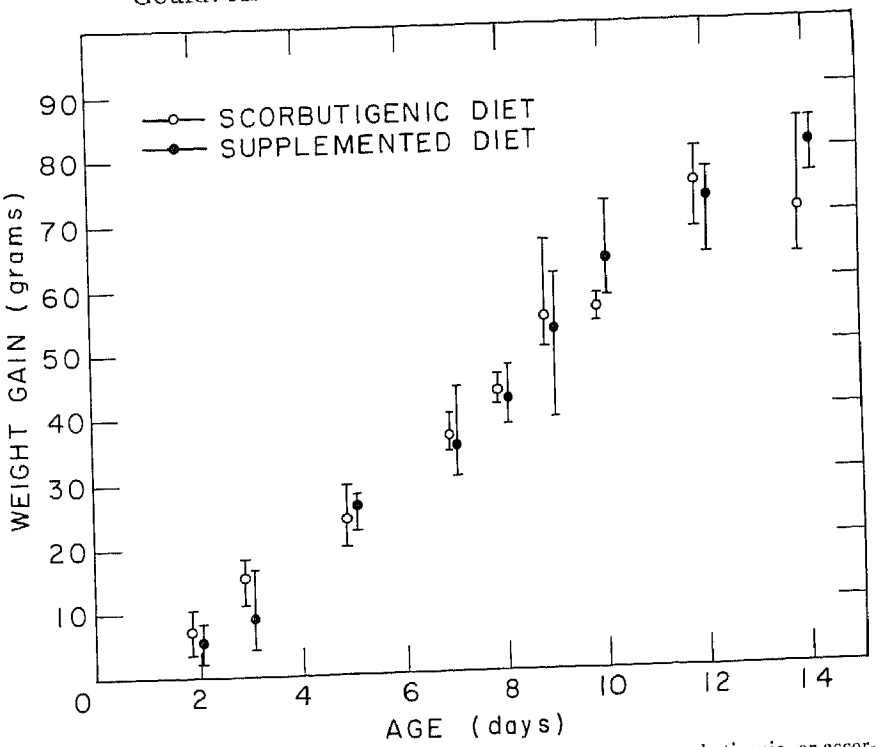

FIGURE 1. Growth of newborn guinea pigs maintained on either a scorbutigenic- or ascorbic acid-supplemented diet. Each point represents the average weight gain of three animals, and the bar represents the range. Each pair of points represents three pairs of litter-mates.

TABLE 1

CHANGES IN TOTAL COLLAGEN CONTENT OF NEWBORN GUINEA PIGS MAINTAINED ON NORMAL AND SCORBUTIGENIC DIETS

	Skin			Carcass		
	Newborn	Supplemented	Scorbutigenic	Newborn	Supplemented	Scorbutigenic
Total collagen (mg.)	471	1390	928	740	1482	1305
Collagen increase/animal (mg.)	—	922	457	—	742	565
Collagen increase (mg.)/gm. tissue increase	—	73.8	83.0	—	10.1	9.7
Collagen (mg.)/gm. tissue*	34	52.6	47.6	18.5	13.1	13.3
Collagen (mg.)/gm. body weight	6.0	6.4	5.3	9.5	6.8	6.8
Soluble collagen (mg.)/gm. insoluble collagen	56.4	159	17.1	17.0	31.8	19.2

Animals were maintained on the scorbutigenic or supplemented diet for 14 to 31 days, and all weighed over 150 gm. Average weight of those on scorbutigenic diet was 173 gm. and on the supplemented diet 217 gm.

After 6 days on scorbutigenic diet, total collagen of skin and carcass was 517 mg. and 840 mg., respectively.

* Either skin or carcass.

rectly related to the amount of growth. Collagen formation in the carcasses of animals on normal and scorbutigenic diets is strikingly similar, and the formation in the skin is slightly higher in animals on the supplemented diet as compared with animals on the unsupplemented diet.

It is quite apparent that salt-soluble collagen was abundant only in the skin of animals on the supplemented diet. However, even though the animals on the scorbutigenic diet had skins relatively poor in salt-soluble collagen, synthesis occurred quite extensively. Rapid collagen synthesis in the carcass of animals either on ascorbic acid-supplemented or scorbutigenic diets was unaccompanied by the accumulation of high concentrations of salt-soluble collagen. There was a slightly higher concentration in the carcasses of normal animals on the supplemented diet, but it was only about one fifth of the relative amount found in the skin of the same animals.

TABLE 2
COLLAGEN FORMATION IN REGENERATING SKIN WOUNDS IN NEWBORN GUINEA PIGS

Days on scorbutigenic diet		Ascorbic acid* after wounding (days)	No. of animals	Collagen† per cent dry weight
Before wounding	After wounding			
0	5	0	9	11.0 ± 2.7
0	6	0	5	31.7 ± 1.1
6	0	6	5	30.6 ± 2.2
6	6	0	4	5.6 ± 1.4
10	6	0	7	4.5 ± 1.0
10	8	0	4	5.4 ± 0.3
12	6	0	3	6.4 ± 1.1

* Ten mg. per day.
† Calculated from total hydroxyproline × 7.46.

These data make it difficult to interpret the significance of large accumulations of salt-soluble collagen. It does not negate the possibility that salt-soluble collagen is the immediate precursor of the fibrous collagen, but it does suggest that in the skin, during rapid growth under normal conditions, soluble collagen may accumulate more rapidly than it can be fibrillated.

Formation of collagen in skin wounds in growing young guinea pigs maintained on scorbutigenic and ascorbic acid-supplemented diets. A number of groups of newborn animals were wounded by removing circles of skin, each three-fourths inch in diameter. In order to determine whether, as had been reported previously for older animals,[5] a depletion period was essential before collagen formation was impaired in newborn animals, one group was wounded on the day of birth and continued on the scorbutigenic diet for 5 and 6 days. When collagen synthesis in this group was compared with a control group that had received an ascorbic acid supplement daily (TABLE 2) it was evident that in all probability these newborn carried over sufficient ascorbic acid from the mother to continue synthesis for a short time.

Another group wounded after a depletion period of six days and then maintained on the scorbutigenic diet no longer formed very substantial amounts of

collagen. Other groups wounded after more prolonged preparative periods also formed only a small amount of collagen. It is apparent therefore that even though the animals in these groups could synthesize substantial amounts of "growth" collagen they had been unable to accumulate "repair" collagen effectively except when ascorbic acid was administered.

It is interesting that wounds that could not repair properly still accumulated a small but significant amount of collagen. It has been assumed in the past that this is collagen that infiltrates the area from the adjacent tissue. It is possible that this small but significant amount of collagen is the product of an ascorbic acid-independent collagen-synthesizing mechanism. It is also possible that this may serve as a foundation upon which the ascorbic acid-dependent collagen-forming mechanism deposits its rapidly produced collagen. This may explain why collagen formation is so rapid in a wound in a deficient animal after ascorbic acid is administered when compared to the formation

TABLE 3

COLLAGEN FORMATION IN SUBCUTANEOUSLY IMPLANTED POLYVINYL SPONGES IN NEWBORN GUINEA PIGS

Treatment	Days					
	8	10	11	12	13	14
	Collagen/sponge (μg.)					
Ascorbic acid-supplemented diet	165	832	938	1125	1130	1365
Scorbutigenic diet	60	173	173	318	338	135

Collagen is based on the hydroxyproline content × 7.46.
Each value is on the average based on 6 animals.
Animals on the supplement received 10 mg. L-ascorbic acid daily.

in a normal animal where the rapid repair phase is preceded by an induction period. These possibilities are presently under investigation.

Formation of collagen in polyvinyl sponges implanted in newborn guinea pigs maintained on scorbutigenic and ascorbic acid-supplemented diets. Several series of newborn guinea pigs were placed on the scorbutigenic diet for a period of six days, at which time four 10-mg. sponges were implanted subcutaneously in each animal. The animals were divided into two groups so that littermates could serve as controls. The animals in one group were maintained on the scorbutigenic diet, and the others received 10 mg. L-ascorbic acid daily. It is evident from TABLE 3 that the animals on the supplemented diet produced an abundance of collagen in the sponges, whereas the animals on the scorbutigenic diet produced considerably less. It is interesting to point out that in the latter some collagen was produced with a tendency for a peak at about 13 days. It is not at all improbable that the small amounts of collagen found when the animals were maintained on the scorbutigenic diet were due to a complex interaction involving both collagen formation and collagen resorption and that in the absence of ascorbic acid resorption kept pace with synthesis.

However, it is clear that the repair mechanism must differ from the mechanism during growth since these animals, during this period of ascorbic acid deprivation, were actively producing growth collagen in the skin and in the carcass.

Summary

The hypothesis that there might be more than one mechanism for collagen biosynthesis, one that is involved in normal body collagen formation and another that predominates in tissue repair, has been tested.

Using newborn guinea pigs it has been demonstrated that body collagen, both in the skin and in the carcass, was extensively formed even when the animals were maintained on a scorbutigenic diet, suggesting that such "growth" collagen is essentially ascorbic acid-independent.

On the other hand, collagen formation in skin wounds or in subcutaneously implanted polyvinyl sponges in these same animals was markedly impaired when the animals were deprived of ascorbic acid, suggesting that "repair" collagen is ascorbic acid-dependent.

Even under the most drastic conditions of ascorbic acid deprivation some collagen was formed in wounds and sponges, and it is suggested that both growth and repair collagen are involved in the repair process and that the former may serve as a foundation upon which the rapidly forming repair collagen accumulates.

There remains the possibility that ascorbic acid is involved in the formation of growth collagen but at a level that is extremely small compared with that for repair collagen.

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