Effect of Ascorbic Acid Deprivation in Guinea Pigs on Skeletal Metabolism [1]

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ABSTRACT The skeletal response to ascorbic acid deficiency in guinea pigs was studied. A reduction in skeletal deposition of both radioactive calcium and phosphorus was consistently observed following 14 days of vitamin C deprivation; and the lability of deposited bone salts was increased in the deficient animals. Subsequent calculations showed a highly significant inverse relationship between these parameters. Development of each was dependent on the time of deficiency. The close relationship between deposition and bone salt release leads to the suggestion that the type and amount of matrix formed was altered by the ascorbic acid deficiency. It is suggested that ascorbate deprivation, similar to that of some other deficiencies, may impede the transition of amorphic bone salts to the crystalline form.

The requirement of ascorbic acid for bone tissue formation has been well-established (1). It appears to influence collagen formation (2) and is also involved in skeletal alkaline phosphatase activity (1). More recent efforts have been directed toward the elucidation of the mode of action involved. Evidence had been obtained which indicated that ascorbic acid was involved in the hydroxylation of proline during the formation of collagen (3). In this light, it has been suggested that ascorbate acts as a hydrogen donor in the hydroxylation reaction (4).

The possibility that vitamin C may influence skeletal calcification, independent of the matrix effect, has also been considered (5). Bourne (6) observed that bone tissue regeneration was dependent on ascorbic acid, and others (7) have noted that skeletal ^{32}P deposition was significantly decreased in scorbutic animals. In the latter case bone was the only tissue of several tested so influenced. Thus, while it is clear that vitamin C is required for bone formation, the means by which it influences this processes remains to be shown.

The amount of bone tissue present at any one time is obviously dependent on a relative balance between bone formation and destructive activities. Another factor which also seems important to bone tissue maintenance is the stability of those crystals which have been formed. In the current study, bone salt deposition and stability were measured in an effort to determine skeletal response to ascorbic acid deficiency.

METHODS

Male guinea pigs weighing 300 to 400 g were used for the first 2 studies. Before the experiments the animals were fed a commercial guinea pig ration [2] and were given a scorbutogenic diet [3] or the same diet supplemented with crystalline ascorbic acid (400 mg/kg) during the experiment. Both feed and water were supplied free-choice during the preliminary and experimental periods. A 15-day feeding period was followed in each experiment.

For the first study, 12 deficient and 12 control animals were divided equally and given either $^{45}CaCl_2$ (10 μCi/100 g body weight) or $H_3{}^{32}PO_4$ (5 μCi/100 g) after 14 days of dietary treatment. The isotopic materials were suspended in physiological saline solution and administered intraperitoneally. After 24 hours the animals were killed; the tibiae were immediately re-

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[2] Purchased from Ralston Purina Company, St. Louis.

[3] This diet had the following percentage composition: ground oats, 40; ground wheat bran, 15; alfalfa meal, 8; skim milk powder (treated to destroy vitamin C), 20; vitamin-free casein, 10; vegetable oil (corn), 5; NaCl, 0.5; $CaCO_3$, 1; and $MgSO_4$, 0.5 (obtained from Nutritional Biochemicals Corporation, Cleveland).

moved, cleaned, and placed in iced physiological saline solution. When cooled, the proximal cancellous region was separated from the compact, blotted with paper towels to remove marrow and apparent moisture, weighed, and placed in 3.0 ml of cool incubation medium (8) which had been adjusted to a pH 7.4 before introduction of the sample. All tissue was incubated for 1 hour in the Dubnoff metabolic shaker at 37°, using air as the gas phase in an attempt to assess the bone salt stability. Following the 1-hour incubation the radioactivity of the medium and the bone tissue was determined and the values were used to calculate the percentage of isotope released during this period. Chemical determinations of similar bone tissue not incubated include citric acid (9) and alkaline phosphatase (10).

A similar procedure was used in the second study with the following exceptions. Radioactive phosphorus (10 μCi/100 g body weight) was given either 12 hours or 6 days before killing (the animals were killed after 15 days of dietary treatment). This was done in an attempt to study the influence of the vitamin C deficiency on stability of bone salts which had been deposited for some time (6-day group) as well as those recently deposited (12 hours). In addition, studies were conducted on inactivated bone tissue. Inactivation was effected by submerging samples in boiling water for 5 minutes before incubation. Food consumption and body weight changes were carefully recorded for all animals used in these experiments.

Next, we wished to determine the amount of time required to cause changes in bone salt deposition and lability when an ascorbate-deficient diet was fed. Thirty young, male guinea pigs were equally divided and given commercial rabbit ration [4] or this feed supplemented with ascorbic acid as in the first 2 studies. This diet was chosen for the present work because the animals consumed it more readily. Previous studies had illustrated that it had scorbutogenic properties comparable to the diet used in the first-described investigations, and it supported an excellent growth rate in guinea pigs when supplemented with ascorbic acid (400 mg/kg). At 4, 9, and 14 days following initiation of dietary treatment, 5 animals from each group were given $H_3{}^{32}PO_4$ (15 μCi/100 g body weight). After 24 hours the animals were killed and observations similar to those of the first 2 studies were made.

Concentration of the skeletal isotopes was determined in the following manner. Bone tissue was ashed overnight at 600°. The resulting ash was taken up with 1 N HCl and a small portion of the liquid sample plated on a stainless steel planchet at infinite thickness. Distribution of the plated sample was enhanced with ethanol. Radioactivity of the medium was measured to determine the degree of skeletal release of isotope during incubation. Media samples were treated in a manner similar to that for the bone ash samples. Both bone ash and media were counted with a gas-flow detector (Nuclear-Chicago) with appropriate calculations (physical decay, etc.) used.

RESULTS

The reduction of bone alkaline phosphate activity in animals given the scorbutogenic diet (table 1) was expected. A number of workers have noted that this change is associated with scurvy, as reviewed by Bourne (1). However, this as-

TABLE 1

Metabolic changes effected by vitamin C deficiency in the guinea pig [1]

Assessment	Control	Scorbutic
Skeletal alkaline [2] phosphatase activity	23.1 ± 0.25 [3]	8.7 ± 0.15 [4]
Bone citric acid concn, μg/100 mg	609 ± 31	605 ± 34
Food intake [5]	100	115
Body wt change, % [6]	+3	−6

[1] Each value represents a mean of 24 animals used in the first 2 experiments.
[2] Refers to the micromoles of *p*-nitrophenyl phosphate split/hour by extract from 100 mg of bone.
[3] Mean ± SE of mean.
[4] Difference between means of scorbutic and control animals is significant ($P < 0.01$).
[5] Intake/unit of body weight; scorbutic value relative to control.
[6] Change in body weight compared to weight when the experiment was started.

[4] Purina Rabbit Chow, Ralston Purina Company. This diet contained the following ingredients of unknown quantities: alfalfa meal, ground yellow corn, dehydrated alfalfa meal, wheat middlings, soybean oil meal, cane molasses, vitamin and mineral supplements; no ascorbic acid was added.

sessment was considered an important addition to this experiment because it provided supporting evidence of the scorbutic condition even though the deficient diet was fed only 15 days. Bone citric acid concentration did not appear to be influenced by the ascorbic acid deprivation which suggested that no influence on bone resorptive function had occurred.

It was evident that quantitative skeletal deposition of both isotopes was inhibited in the deficient animals (tables 2, 3, and 4) which substantiates the results of Friberg and Ringertz (7). Evidence has also been presented suggesting that the rate of skeletal calcium deposition is impeded by this deficiency (11). Skeletal deposition of the isotopes appeared to be inversely related to the length of time of the deficiency (table 4). A calculation of the correlation coefficient between time of deficiency and skeletal deposition (12) (table 4) showed a highly significant value ($r = -0.900$).

Lability, which was estimated by measuring the release of the isotope from the bone to the medium during incubation, was consistently and significantly greater for bone tissue from deficient animals (tables 2, 3 and 4). This was true for both isotopes (table 2), for isotopes deposited for some time or only recently (table 3), in both live and deactivated tissue (table 3), and was influenced by the amount of time the animals were fed the scorbutogenic diet (table 4). In the latter case (table 4)

TABLE 2

Influence of ascorbic acid deficiency on skeletal mineral deposition and release in the guinea pig (12 animals/group)

Assessment	Control	Scorbutic
Skeletal ^{45}Ca [1] activity [2]	21.7 ± 1.4 [3]	11.8 ± 0.9 [4]
Skeletal ^{45}Ca released [5]	0.25 ± 0.03	0.57 ± 0.10 [4]
Skeletal ^{32}P [6] activity	15.5 ± 0.7	9.4 ± 0.6 [4]
Skeletal ^{32}P released	0.62 ± 0.02	1.53 ± 0.16 [4]

[1] Administered intraperitoneally as $^{45}CaCl_2$ (10 μCi/100 g body wt).
[2] Counts/minute × 10^3 per mg of bone ash.
[3] Mean ± SE of mean.
[4] Differences between means of scorbutic and control animals is significant ($P < 0.01$).
[5] Percentage of total skeletal isotope content released during incubation.
[6] Administered intraperitoneally as $H_3{}^{32}PO_4$ (5 μCi/100 g body wt).

TABLE 3

Additional evidence concerning skeletal deposition and release of radioactive minerals (6 animals/group)

Assessment	Control	Scorbutic
Isotope administered 6 days before killing		
Skeletal ^{32}P concn [1]	59.0 ± 10.2 [2]	24.8 ± 2.7 [3]
Skeletal ^{32}P released [4]	0.17 ± 0.03	0.42 ± 0.06 [3]
Skeletal ^{32}P released [5]	0.05 ± 0.01	0.13 ± 0.04 [6]
Isotope administered 12 hours before killing		
Skeletal ^{32}P concn [1]	61.5 ± 10.8	18.1 ± 2.0 [3]
Skeletal ^{32}P released [4]	0.55 ± 0.04	1.85 ± 0.23 [3]
Skeletal ^{32}P released [5]	0.21 ± 0.04	0.54 ± 0.14 [6]

[1] Counts/minute × 10^3/mg of ash. Isotope administered intraperitoneally as $H_3{}^{32}PO_4$ (10 μCi/100 g body wt).
[2] Mean ± SE of mean.
[3] Difference between means of scorbutic and control animals is significant ($P < 0.01$).
[4] Percentage of isotope released from bone to medium by live bone tissue/hour.
[5] Percentage of isotope released from bone to medium by inactivated bone tissue/hour.
[6] $P < 0.05$.

TABLE 4

Development of skeletal deficiency symptoms in the guinea pig

Days of dietary treatment	Group	Food intake [1]	Wt gain (cumulative)	Skeletal ^{32}P [2]	^{32}P released [3]
			g		
5	Control	100.0	22 ± 3 [4]	86 ± 29	0.50 ± 0.08
	Scorbutogenic	123.4	25 ± 3	91 ± 23	0.50 ± 0.05
10	Control	100.0	43 ± 9	90 ± 8	0.62 ± 0.03
	Scorbutogenic	100.2	39 ± 12	61 ± 10 [5]	0.74 ± 0.10
15	Control	100.0	59 ± 5	86 ± 5	0.64 ± 0.03
	Scorbutogenic	98.4	48 ± 17	42 ± 7 [5]	1.01 ± 0.14 [5]

[1] Scorbutogenic values relative to control. Each group (5, 10, and 15 days) was composed of 5 animals for each dietary treatment.
[2] Bone ^{32}P concentration in counts/minute × 10^3/mg ash. Isotope was administered intraperitoneally as $H_3{}^{32}PO_4$ (15 μCi/100 g body wt).
[3] Percentage of the total skeletal activity released from the bone to the medium during a 1-hour incubation period.
[4] Mean ± SE of mean.
[5] Difference between means of scorbutic and control animals is significant ($P < 0.01$).

the degree of lability was increased as deposition decreased, which suggested that the 2 factors were inversely related. A calculation of the correlation coefficient (12) between these factors showed a significant association ($r = -0.785$).

Food intake (tables 1 and 4) was measured in case inanition was an influential factor. The results suggested that the deficient group consumed considerably more feed during the early stages of the deprivation period (table 4) and was comparable to that of controls during the latter phase of the experiment. Despite the greater intake of the scorbutogenic diet, the animals failed to gain as much weight as controls (tables 1 and 2); however, the weight gain differences were not statistically significant.

DISCUSSION

The expected bone lesions in scurvy are generally considered to be a consequence of an impaired function of the osteoblast. This implies that the calcification processes remain unchanged in this deficiency state; or, if changed, such alterations result from a malformed or insufficient amount of matrix.

It was clear that skeletal deposition of the injected isotopes was reduced in the ascorbic acid-deficient animals (tables 2, 3, and 4). It does not seem logical to attribute this result to an increased resorption rate because no change in bone citric acid occurred (table 1). Neuman et al. (13) demonstrated directly that parathyroid hormone enhanced the production of citric acid by bone; and Mecca et al. (14) found that the addition of this hormone extract to organ cultures stimulated the output of citrate by bone tissue. Further, the effect was evident and as extensive in animals injected 12 hours before killing as it was in those given the isotope 6 days before (table 3). Finally, these results agree with earlier observations (15) that the osteoclast population did not increase in scurvy although evidence of bone rarefaction was evident.

It appears most probable that the reduced isotope deposition (tables 2, 3, and 4) in the vitamin C-deficient animals was partially due to an impedance of osteoblastic activity. Direct results have been obtained showing that ascorbic acid is required for collagen formation (3) and many others have provided indirect evidence (1). Additionally, some of the observations and calculated relationships which were made in this work suggest other implications. The decreased stability of the bone salts formed in the deficient animals (tables 2, 3, and 4) appears to be of consequence. Since isotope deposition and lability were inversely related and time-dependent in development, it is possible that not only quantity but also matrix quality was influenced by the deficiency. Although no direct assessments were made regarding the nature of the bone salts deposited, certain inferences can be made based on the results of others. Harper and Posner (16) have shown quantitatively that bone tissue contains amorphous calcium phosphate as a second major mineral phase in addition to crystalline apatite. Termine and Posner (17) found that rickets was associated with a decrease in the crystalline apatite fraction and a subsequent increase in the amorphic phase. Since deficiencies of vitamin D, calcium, and phosphorus had a similar impact (17), it follows that the response was general.

Generally it is belived that amorphic solids are more reactive chemically than crystalline solids; thus, it appears that amorphism would be associated with increased lability. The in vitro results of this study were consistently suggestive of increased lability in vitamin C-deficient bone tissue (tables 2, 3, and 4). Perhaps this particular deficiency (ascorbic acid) is similar to vitamin D, calcium, or phosphate deprivation (17) in that the metastasis of bone salts from the amorphic to crystalline phases is impeded by its absence.

LITERATURE CITED

1. Bourne, G. H. 1956 The Biochemistry and Physiology of Bone. Academic Press, New York, p. 551.
2. Gould, B. S., and J. F. Woessner 1957 The influence of ascorbic acid on the proline, hydroxyproline, glycine, and collagen content of regenerating guinea pig skin. J. Biol. Chem., 266: 289.
3. Gould, B. S. 1958 Biosynthesis of collagen. III. The direct action of ascorbic acid on hydroxyproline and collagen formation in

subcutaneous polyvinyl sponge implants in guinea pigs. J. Biol. Chem., *232:* 637.

4. Peterkofsky, B., and S. Udenfriend 1965 Enzymatic hydroxylation of proline in microsomal polypeptide leading to formation of collagen. Proc. Nat. Acad. Sci., *53:* 335.
5. Bourne, G. H. 1943 Some experiments on the possible relationship between vitamin C and calcification. J. Physiol., *102:* 319.
6. Bourne, G. H. 1942 The effect of ascorbic acid (vitamin C), calcium ascorbate and calcium gluconate on the regeneration of bone in rats. Quart. J. Exp. Physiol., *31:* 319.
7. Friberg, U., and N. R. Ringertz 1954 Uptake of radiosulphate and radiophosphate in various tissues of normal and scorbutic guinea pigs. Exp. Cell Res., *6:* 527.
8. Thornton, P. A., and J. L. Omdahl 1966 The influence of age on skeletal response to immobilization and ascorbic acid deficiency. Proc. 7th International Congress of Gerontology, Vienna, Austria.
9. Pucher, G. W., C. C. Sherman and H. B. Vickery 1936 A method to determine small amounts of citric acid in biological material. J. Biol. Chem., *1113:* 235.
10. Lowry, O. H., N. R. Roberts, M. L. Wu, W. S. Hixon and E. J. Crawford 1944 The quantitative histochemistry of brain. II. Enzyme measurements. J. Biol. Chem., *207:* 19.
11. Manoussos, G., and G. Milhaud 1958 Effet de la carence en vitamine C sur le metabolisme du calcium chez le cobaye. Compt. Rend., *247:* 2481.
12. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Ames, p. 174.
13. Neuman, W. F., H. Firschein, P. S. Chem, Jr., B. J. Mulryan and V. Distefano 1956 On the mechanism of action of parathormone. J. Amer. Chem. Soc., *78:* 3863.
14. Mecca, C. E., G. R. Martin and P. Goldhaber 1963 Alteration of bone metabolism in tissue culture in response to parathyroid extract. Proc. Soc. Exp. Biol. Med., *113:* 538.
15. Hess, A. F. 1920 Scurvy past and present (cited in Bourne, G. H. 1956 The Biochemistry and Physiology of Bone. Academic Press, New York, p. 556).
16. Harper, R. A., and A. S. Posner 1966 Measurement of non-crystalline calcium phosphate in bone mineral. Proc. Soc. Exp. Biol. Med., *122:* 137.
17. Termine, J. D., and A. S. Posner 1967 Amorphous/crystalline interrelationships in bone mineral. Calcified Tissue Res., *1:* 8.