Further Evidence of Skeletal Response to Exogenous Ascorbic Acid[1] (34272)

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The mobilization of previously deposited ^{45}Ca from the chick's skeleton appeared to be increased by exogenous ascorbic acid (1). As reviewed in that report, it is accepted that ascorbate plays a role in bone formation (2); but its implication in bone destruction has not been demonstrated, other than the just-mentioned case. The current work concerns an effort to re-investigate the bone salt mobilization possibility in greater depth.

Acid phosphatase activity of the plasma was assessed because of its relationship to lysosomal enzymes and the findings which link these enzymes to the destructive phase of bone and cartilage, as illustrated by parathyroid hormone and vitamin A studies (3–5). Ascorbic acid may also act similarly since esterase and protease activities are lower in scorbutic guinea pigs (6). Further, it has been reported that excess ascorbic acid elevated liver lysosomal enzyme levels and altered their distribution (7). Ascorbic acid also appears to enhance connective tissue destruction when administered to scorbutic guinea pigs (8). Finally results from this laboratory (9, 10) indicated that ascorbic acid altered bone cellular energy metabolism and also appeared to enhance the release of ^{45}Ca and acid phosphatase from bone studied in tissue culture. In view of these reports concerning the possible ascorbate implication on lysosomal activity and the earlier work which associated ascorbate with bone ^{45}Ca mobilization (1), it was of interest to see if the factors were altered simultaneously in the presence of this compound.

Materials and Methods. Four separate studies were conducted using a similar strain of male leghorn chicks. These animals were given a complete diet from 1 day of age until the studies were terminated. Composition of the diet was as follows: yellow corn meal, 68.6; soybean oil meal (50% protein), 25.0; dehydrated alfalfa meal (17% protein), 1.5; limestone, 2.4; dibasic sodium phosphate, 2.0; and sodium chloride, 0.5, all expressed as percentage of the diet. Manganese sulfate (70%) was added at 220 mg/kg, and a vitamin mix previously used (11) was included.

In Expts. A and B (Table I) the chicks were given $^{45}CaCl_2$ (A $\doteq$ 10 μCi/100 g, B = 20 μCi/100 g of body wt) intramuscularly (im) when 11 days of age. The isotope was suspended in 0.9% saline with its concentration regulated so that the animals in each experiment received equal volumes per unit of body weight. When 15 days old, the chicks were placed into experimental groups (Table I) and injected intraperitoneally (ip) with either normal saline or this material containing ascorbic acid (reagent grade L-ascorbic acid). The ascorbate crystals were dissolved in the saline solution just prior to administration and given at levels indicated in Table I. Comparable volumes of solution (0.5 ml/100 g of body wt) were given in all cases. Experiment C (Table I) was similar to A and B in all respects with the exception that no ^{45}Ca injections were made.

In each case (A through C) blood samples were taken by cardiac puncture 4 hr following the ip injection of the test materials. The samples were collected in heparinized syringes, deposited in chilled centrifuge tubes and immediately centrifuged at approximately 2500 rpm until separation of the plasma had occurred. All samples were kept cold

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TABLE I. Plasma Changes Following Ascorbate Injection.

Injection		Acid phosphatase (μmoles/ml/30 min)[a]	^{45}Ca (cpm/ml)[b]
Expt. A			
Saline, 0.9%	(9)[c]	0.91 ± 0.02[e]	81 ± 4
+ Asa, 5 mg[d]	(9)	1.06 ± 0.05[g]	92 ± 14
10 mg	(9)	1.15 ± 0.08[g]	107 ± 4[g]
20 mg	(9)	1.36 ± 0.12[g]	114 ± 4[g]
Expt. B			
Saline, 0.9%	(10)	0.99 ± 0.04	187 ± 14
+ Asa, 10 mg	(10)	1.21 ± 0.06[g]	221 ± 10[f]
Expt. C			
Saline, 0.9%	(8)	0.92 ± 0.05	
+ Asa, 5 mg	(8)	1.04 ± 0.03[f]	
10 mg	(8)	1.19 ± 0.04[g]	
20 mg	(8)	1.40 ± 0.22[f]	

[a] Micromoles of *p*-nitrophenyl phosphate hydrolyzed per ml of plasma per 30-min incubation time.

[b] Corrected by bone ^{45}Ca activity; see "Methods and Materials" for full explanation.

[c] Number of animals per group is given in parentheses.

[d] Refers to the level of ascorbic acid given/100 g of body weight.

[e] Mean ± the standard error of the mean.

[f g] Significantly different from the control group at the 5 and 1% confidence levels, respectively.

until the portion for acid phosphatase activity determination was taken. This assessment was completed within 1 hr following blood collection, using a slight modification of the Lowry *et al.* method (12). The only change was the use of an acetate buffer. Plasma was added to the buffer–substrate mixture and the procedure continued as before (12).

Plasma ^{45}Ca activity was determined as described earlier (1). This value was normalized by the level of skeletal ^{45}Ca activity (cpm/mg of ash, using compact tissue from the tibia). Under these experimental conditions this correction seems pertinent. The isotope was given several days prior to the experiment. Usually individuals vary somewhat in the amount of isotope deposited in the bone as well as in relative turnover rates. Individual differences in the skeletal ^{45}Ca content could lead to erroneous results when comparisons are made between groups. This is based on the assumption that blood ^{45}Ca activity is related to skeletal ^{45}Ca activity. Subsequent analysis revealed that these factors were highly correlated (13) in both control and rachitic chicks ($r > 0.8$ in all groups). The results also held for both compact and metaphyseal bone taken from animals given a control or rachitogenic diet.

The final study (Table II) was conducted similarly to the ones just described with the following exceptions: no analysis for acid phosphatase was made, and total plasma calcium was determined with an atomic absorption spectrophotometer (Instrumentation Laboratories model 153). Two separate experiments, involving 7 chicks/group in each case, were conducted. Since the results were similar, the data were combined into 1 value (Table II).

Results and Discussion. Plasma acid phosphatase activity was consistently elevated following ascorbate injection. This response appeared to increase with dose within the levels used in these studies. The physiological meaning of these observations is not immediately apparent. If lysosomal enzymes are associated with bone resorptive function, then it follows that the increased acid phosphatase (Table I) may have reflected a stimulation in bone breakdown, providing the enzyme originated in bone cells and not from other

TABLE II. Ascorbate and Plasma Calcium.

Injection	Calcium (μg/ml)	^{45}Ca (cpm/μg of calcium)	^{45}Ca (cpm/μg of calcium) (bone corrected)[a]
Saline, 0.9% (14)[b]	102 ± 3[c]	5.8 ± 0.3	2.2 ± 0.1
Saline, 0.9% Asa, + 10 mg[d] (14)	93 ± 3[e]	7.4 ± 0.4[f]	2.7 ± 0.2[e]

[a] See footnote *b*, Table I.
[b] See footnote *c*, Table I.
[c] See footnote *e*, Table I.
[d] See footnote *d*, Table I.
[ef] Significantly different from the control group at the 5 and 1% confidence levels, respectively.

tissue cells. Supporting such a possibility are the data which indicated that parathyroid extract (4) as well as vitamin A (5), both inducers of bone breakdown, enhanced lysosomal enzyme release from bone *in vitro*. Additionally, the evidence that ascorbic acid also appeared to increase the movement of acid phosphatase from bone *in vitro* (10) points to its having a similar influence.

Plasma ^{45}Ca activity (cpm/ml) (Table I) was also increased as the level of injected ascorbate was increased. This elevation was very much like that observed for plasma acid phosphatase changes. A correlation analysis (13) between these 2 factors revealed a highly significant relationship ($r = 0.960$; $p > 1\%$). From these results and an earlier report (1) it is suggested that exogenous ascorbic acid, when given to the chick, was associated with a mobilization of previously deposited skeletal ^{45}Ca. Whether ^{45}Ca was released from newly laid down bone or older bone cannot be determined from these data. Further, the possibility exists that the effect could have been either a direct or indirect response to the administered ascorbate. The evidence that plasma acid phosphatase rose at a similar rate to ^{45}Ca suggests that this factor was mutually influenced, although its origin could have been from other tissues.

Results from the last study (Table II) support the earlier observations in that plasma ^{45}Ca rose following ascorbate injection. Additionally, the total plasma calcium was significantly lower in the experimental animals. A possible explanation for these seemingly paradoxical observations (hypocalcemia evidence of bone calcium mobilization) may be as follows. The initial response to the ascorbate (direct or indirect) was a decrease in plasma calcium. This response would seemingly be followed by compensatory bone salt mobilization. Since the plasma ^{45}Ca activity was elevated, it follows that either the proposed skeletal site of mobilization was an area of relatively intense ^{45}Ca concentration; or a greater portion of the plasma calcium pool could be attributed to skeletal origin following ascorbate administration. Assuming that the plasma ionic calcium level was decreased during the hypocalcemic response, it follows that parathyroid hormone output was probably increased. In such case, it may be that the plasma changes, indicative of bone salt mobilization, were effected by this hormone, relegating the ascorbate to an indirect role.

Just why ascorbic acid administration was associated with a drop in total plasma calcium is not clear. Its apparently weak chelating properties (14) would seemingly suppress that possibility. Although some data have indicated that ascorbate may increase intestinal absorption of calcium (15), there seems to be no evidence that it may inhibit this function. The influence of ascorbate on the renal handling of calcium has apparently not been studied. Whether any of these functions are involved must remain in doubt for now. Regardless, it remains that exposure to exogenous ascorbic acid was followed by a rise in plasma ^{45}Ca and acid phosphatase activities. Alone and collectively, these factors are indicative of bone resorption.

Summary. The response to ascorbic acid administration was studied using the young

chick. Plasma acid phosphatase and ^{45}Ca activities (^{45}Ca previously incorporated by the skeletal system) were consistently and equally elevated following ascorbate injection. Each of these factors is indicative of bone resorptive activity. Since total plasma calcium was depressed by the ascorbate, it is suggested that the effect on the skeleton may have been indirect, however.

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