

# Ascorbic Acid and the Calcium Metabolism of Embryonic Chick Tibias<sup>1</sup> (35559)

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Abnormal connective tissue is a primary lesion of ascorbic acid deficiency. The best documented role for the vitamin is its requirement in collagen biosynthesis both *in vivo* (1-3) and *in vitro* (4-7). It has also been shown that vitamin C has a stimulatory effect on aerobic energy metabolism in cultured bones (8). Although the foregoing studies attest to the importance of ascorbic acid in bone matrix production and energy metabolism, little is known concerning its effect on bone mineralization. Slater and Aub (9) showed that a scorbutic diet inhibited deposition of calcium in bone, but they did not show that vitamin C stimulated calcification. Similarly, Bourne (10) indicated that less bone salt was laid down by normal as well as injured bone in scorbutic animals compared to controls. However, both of these findings might simply have been due to a lack of calcifiable matrix.

The present study was undertaken to ascertain if ascorbic acid has an effect on mineral mobilization from cultured bones. Embryonic chick tibias prelabeled with radiocalcium were allowed to become "scorbutic" in tissue culture (7, 8). Ascorbic acid-dependent release of <sup>45</sup>Ca from these bones was assessed in terms of the time required to elicit a response after exposure to the vitamin and the longevity of the response once it was elicited. The ratios of inorganic to organic components (% ash) after exposure to the vitamin for various times were also compared.

*Materials and Methods. Tissue culture.* Embryonated, white leghorn eggs were injected on day 17 of incubation with a <sup>45</sup>Ca

solution of high specific activity; a small hole was drilled in the shell over the air space, and the isotope solution was deposited directly against the shell membrane with a small syringe. A level of 5  $\mu$ Ci of <sup>45</sup>Ca dissolved in 0.1 ml of physiological saline solution (PSS) was used/egg. After the injection, the hole in the egg was covered with a small piece of surgical tape. On day 18 of incubation, the diaphyseal portions of the tibias were harvested from the embryos, washed in modified Krebs-Ringer phosphate-buffered saline (PBS), and cultured for 5 days in spinner bottles containing 50 ml of chemically defined culture medium. Each bottle contained 48 bone halves and the culture medium was changed at the beginning of days 3 and 5. The details of this tissue culture technique may be found elsewhere (8).

*Short-term incubations.* Immediately following tissue culture, the bones were thrice rinsed in PBS and transferred to 25-ml Erlenmeyer flasks containing 4 ml of PBS plus 100 units-100  $\mu$ g of penicillin-streptomycin. The tibias from any one culture were divided into six samples of eight bones each. All of the short-term incubations were performed in a Dubnoff metabolic shaker at 38° using air as the gas phase and 120 oscillations/min. Both active and inactive release of <sup>45</sup>Ca from the bone to the incubation medium was measured on the same samples during two consecutive 2-hr Dubnoff incubations. The first incubation was done with the tissue transferred directly from tissue culture and the second was after exposing the bones to 120° for 12 hr.

*Analytical methods.* All weighing was done on an analytical balance (Mettler Instrument Corp., Model B-6, Highstown, N. J.) which was read to the nearest 0.1 mg. Drying was

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accomplished by heating the bones at 120° for 24 hr. The tissue was ashed at 600° for 12 hr. Both ashed and dried bones were allowed to equilibrate with room air moisture for 12 hr before weighing. Percentage ash was calculated from the ratio of ash weight to dry weight. The dry weight also served as the basis for the  $^{45}\text{Ca}$  data.

Radioactivity of the samples was assessed with a gas flow detector (Nuclear Chicago Corp., Model D47, Des Plaines, Ill.). Incubation and culture medium samples (0.2 to 0.5 ml) were plated on aluminum planchets, spread with 70% ethanol and dried at 110°. Bone ash samples were dissolved in 2 ml of 1 N HCl; diluted tenfold with distilled water; and 0.1-ml samples were plated, spread, and dried on stainless steel planchets as described above.

**Experimental procedures.** In the first experiment, the release of  $^{45}\text{Ca}$  from four cultures of prelabeled bones was measured during the final 24 hr of culture and during the subsequent active and inactive short-term incubations. After 4 days of culturing without the vitamin, two of the cultures received a single 50  $\mu\text{g}/\text{ml}$  addition of freshly prepared ascorbic acid at the beginning of the final 24 hr of culture. This level of medium ascorbic acid was the same as for several previous tissue culture studies (6-8, 11). The other two cultures served as controls. The second experiment followed a similar protocol except that the short-term incubation was initiated 8 hr after addition of the vitamin on culture day 5. In both experiments, no ascorbic acid was added to the Dubnoff medium. Thus, both ascorbic acid-treated and nontreated bones were exposed to the same medium during the short-term incubations.

**Results.** Increased rate of release of  $^{45}\text{Ca}$  into the culture medium by both control and ascorbic acid-treated tibias was delayed during day 5 of culture (Fig. 1). However, this delay was considerably shortened when ascorbic acid was present in the culture medium. The enhanced  $^{45}\text{Ca}$  mobilization from the control bones occurred 12 to 24 hr after the medium was changed on day 5. When ascorbic acid was added, this lag was decreased to the 6- to 12-hr interval. Sometime during the 12- to 24-hr period, the  $^{45}\text{Ca}$  release rate by

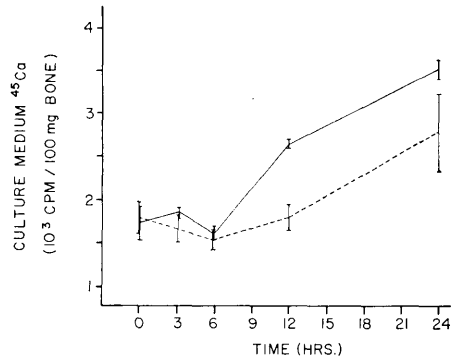


FIG. 1. Release of  $^{45}\text{Ca}$  from labeled tibias after addition of ascorbic acid on day 5 of culture: (each line) represents the mean  $\pm$  the standard error of the mean of serial samples taken from two cultures. Zero time samples were taken just before adding the ascorbic acid. The vitamin was not added until 30 min after the medium was changed on day 5. Before changing the medium, all bones were quickly rinsed with PBS to remove any residual  $^{45}\text{Ca}$  from the previous culture medium; control cultures (---); cultures that received ascorbic acid (—).

both control and treated bones became about equal as shown by the similar line slopes during this time (Fig. 1) and by the release data obtained from the subsequent short-term incubation (Table I). Neither the active nor the heat-inactivated  $^{45}\text{Ca}$  mobilization rate was affected by ascorbic acid after 24-hr exposure to the vitamin. That the ascorbic acid-dependent, transiently augmented mobilization of  $^{45}\text{Ca}$  occurred between 6 and 12 hr after addition of the vitamin is indicated by the results of the short-term incubation performed 8 hr after vitamin treatment (Table I). These data also demonstrate that the ascorbic acid influenced only the active portion of the release. These bones were somewhat less radioactive than those in the first experiment and showed correspondingly lower  $^{45}\text{Ca}$  release rates. Although calcium mobilization seems to be affected by ascorbic acid with a short exposure to the vitamin, percentage ash was not influenced by vitamin C (Table I).

**Discussion.** The initial appearance of  $^{45}\text{Ca}$  in the culture medium of the control bones probably represents a rapid exchange of stable calcium in the medium with radioactive calcium on the surface of the bone mineral (Fig. 1). The culture medium contained

TABLE I. Effects of Ascorbic Acid on Bone Calcium Mobilization with the Vitamin Present for Either the Final 8 or 24 hr of Culture.

	<sup>45</sup> Ca release <sup>a</sup> (10 <sup>3</sup> cpm/100 mg/hr)			Bone <sup>45</sup> Ca content (10 <sup>6</sup> cpm/100 mg)	% Ash
	Total	Inactive	Active (total—inactive)		
Control	4.95 ± 0.92 <sup>b</sup>	3.32 ± 0.08	1.62 ± 0.14	1.21 ± 0.02	47.6 ± 0.2
+ASA, 8 hr <sup>c</sup>	5.96 ± 0.14 <sup>c</sup>	3.25 ± 0.09	2.71 ± 0.15 <sup>e</sup>	1.19 ± 0.01	48.0 ± 0.3
Control	10.4 ± 0.2	6.25 ± 0.21	4.12 ± 0.27	1.32 ± 0.02	49.9 ± 0.3
+ASA, 24 hr <sup>d</sup>	10.5 ± 0.4	6.66 ± 0.31	3.87 ± 0.36	1.36 ± 0.02	49.4 ± 0.4

<sup>a</sup> The release data were obtained from short-term incubations following tissue culture.

<sup>b</sup> Each value is the mean ± standard error of the mean of 12 individual determinations.

<sup>c</sup> Ascorbic acid was added for only the final: 8 hr of culture; <sup>d</sup> 24 hr of culture.

<sup>e</sup> Level of significance ( $p < 0.01$ ) compared to controls.

1.8 mM calcium (8). This exchange would have decreased the specific activity of calcium at the mineral surface and would have occurred irrespective of any metabolic intervention. The 12-hr delay in increased rate of <sup>45</sup>Ca release may represent the time needed to remove this new, lower specific activity mineral and to allow exposure and loss of older, higher specific activity calcium through the turnover of bone mineral. It is suggested that this turnover was dependent on the metabolic activity of the bone cells.

The earlier effect of ascorbic acid on the <sup>45</sup>Ca release at about 6 hr instead of 12 hr supports the latter statement (Fig. 1). Ascorbic acid has previously been shown to cause increased oxidative energy metabolism in bone when the vitamin is present in tissue culture for either 5 days or for only the fifth and final day of culture (8). It is possible that this increased metabolic activity might have been coupled to an augmented turnover rate of mineral in the treated bones resulting in the earlier appearance of higher specific activity calcium via the mechanism described above.

The short-term incubation data further suggest that bone mineral of higher calcium specific activity was eventually exposed after changing the culture medium on day 5 (Table I). The inactive <sup>45</sup>Ca release was nearly doubled at 24 hr compared to the release at 8 hr. This doubling could not have been due to the difference in <sup>45</sup>Ca content of the tibias since the isotope content of the 24-hr bones was <10% higher than that of the 8-hr bones.

It should be pointed out that release of <sup>45</sup>Ca to the short-term medium was not the result of isotope exchange since no calcium was added to the PBS the tibias were incubated in.

Previous work has shown that ascorbic acid decreases the percentage ash value in tibias exposed to the vitamin for 5 days (12). This observation probably resulted from increased matrix content since it has been well established that vitamin C stimulates collagen synthesis in cultured bones (7, 13). Since no changes in percentage ash were obtained in the present study (Table I), it would seem that the ascorbic acid-dependent effects on bone mineral were independent of matrix synthesis. However, it is possible that the turnover of mineral was accompanied by a simultaneous turnover of matrix. If this were the case, the ratio of inorganic to organic component could have remained constant even though net changes in both components were taking place.

Bone remodeling is an orderly, progressive mechanism that encompasses both bone formation and destruction. These processes are essential for the storage and release of calcium and are intimately involved in the highly regulated homeostatic control of blood calcium. Results of this study, combined with previous results by these and other authors, support the hypothesis that ascorbic acid can elicit a bone remodeling response.

*Summary.* Previous work in this laboratory and by others has shown that ascorbic acid is involved in bone matrix production and

energy metabolism. However, scant information is to be found concerning a possible involvement of the vitamin in bone mineral metabolism. The present study was undertaken to ascertain if ascorbic acid has an effect on  $^{45}\text{Ca}$  mobilization from cultured chick tibias. Tibias from 18-day embryos were labeled with  $^{45}\text{Ca}$  *in ovum* and cultured for 5 days in chemically defined culture medium. Half the cultures received a single dose of ascorbic acid (50  $\mu\text{g}/\text{ml}$ ) on day 5. Release of  $^{45}\text{Ca}$  from the bones during tissue culture and during subsequent short-term incubations was assessed. The results of this study showed that ascorbic acid increased calcium turnover in bone, possibly through increased metabolic activity of the bone cells. The response was transient as the rate of  $^{45}\text{Ca}$  release in the treated bones eventually returned to the control level.

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