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Carbonic Anhydrase and the Precipitation of Apatite

Abstract. On theoretical grounds it is unlikely that the catalytic action of the enzyme carbonic anhydrase would be required for the precipitation of apatite in vitro. The presence of carbonic anhydrase in either active or inactivate form did not initiate precipitation in a metastable calcifying solution. It is unlikely that carbonate (or bicarbonate) ions are essential for the precipitation of apatite in vitro or in vivo.

In a recent paper by McConnell *et al.* the conclusion was drawn: ". . . that the carbonate ion is essential to precipitation of bone mineral and that the principal biochemical catalyst in vivo is carbonic anhydrase" (*1*). This conclusion was based on results obtained in experiments in which apatite was deposited in vitro on glass plummets immersed intermittently into saliva or into solutions containing sodium phosphate and calcium chloride. It was claimed that under their experimental conditions (which were not specified in sufficient detail by the authors) the catalytic activity of carbonic anhydrase was necessary to elicit this precipitation. In view of the known properties of this enzyme, their interpretation of the experimental results seems surprising.

The uncatalyzed hydration of CO_2 to carbonic acid is an extremely rapid process (*2*). Hence rapid recording methods are required to demonstrate the enzymatic activity of carbonic anhydrase (*3*). The uncatalyzed reaction goes to completion in a matter of a few minutes.

In mammals the enzyme is found particularly in tissues and cells (for example, red blood cells, stomach wall, kidney tubules, pancreas) where large amounts of CO_2 must be hydrated in fractions of a second, and the speed of the uncatalyzed reaction would be insufficient (*4*). This situation clearly does not exist in the experiments of McConnell *et al.*, in which it took up to 5 days to develop a precipitate of calcium phosphate. The CO_2 molecules slowly diffusing into the solution from the outside atmosphere do not require the activity of the enzyme over such an extended period, particularly not at a *p*H as high as 7.5.

Another interpretation of the results of McConnell *et al.* suggested itself. Carbonic anhydrase is known to be strongly adsorbed to glass surfaces (*5*), and it is conceivable that the added enzyme, in either active or inactivate form, was adsorbed to the glass plummets and became firmly attached to them during the intermittent immersions. The adsorbed carbonic anhydrase might then have caused the deposition of apatite in a way unrelated to its activity in the hydration of CO_2. Adsorbed films can subsequently be calcified by placing them in a calcifying solution. Such plaques can be produced on filter paper disks by intermittent immersion into saliva (*6*).

In order to test whether carbonic anhydrase will initiate deposition of apatite we performed a number of experiments with a metastable calcifying solution which does not deposit any solid in 2 or 3 days, unless initiators like apatite or collagen are present (*7*). The solutions contained the following concentrations (in millimoles) of ions: Na, 145; Cl, 133; total carbonate, 22; K, 5; Ca, 3.75 (150 mg/l); phosphate, 1.6 (50 mg of P per liter). The ionic strength was approximately 0.16. After first bubbling CO_2 through the solution in order to lower the *p*H to about 6.0 and thus prevent spontaneous precipitation upon addition of the calcium solution, we then removed the excess CO_2 by shaking and in this way adjusted the *p*H to 7.3 ± 0.05. Quantities up to 1 mg of the crystalline enzyme (Sigma Chemical Co.) were placed in 50 ml glass erlenmeyer flasks. Then the flasks were filled with the calcifying solution and closed with paraffinized cork stoppers held in place by rubber bands. After 3 days' incubation at 37°C no precipitation had occurred. Even after 1 week the activity of the enzyme, as measured by the method of Philpot and Philpot (*8*), had not decreased. In other experiments as much as 1 mg of the enzyme was dissolved in small amounts of distilled water in the erlenmeyer flasks, and the solutions were evaporated to dryness in an oven at 110°C. This treatment completely inactivated the enzyme. The flasks containing the inactivated enzyme were filled with calcifying solution. As in the case of the active enzyme, no precipitation occurred during 3 days' incubation at 37°C.

When the calcification experiments were carried out for periods longer than 3 days a film gradually formed on the surface of the solutions, even in flasks containing only calcifying solution. This film consisted mainly of apatite (determined from the chemical composition and the x-ray diffraction pattern). It also contained some organic material of bacterial and fungal origin. The addition of a crystal of thymol, a drop of chloroform, or up to 5 mg of sulfanilamide to the calcifying solutions did not prevent the formation of this film. Therefore, it is thought likely that the effect was due to a slow loss of CO_2 through the stoppers or to nucleation in the surface layer of the solution, rather than to the presence of microorganisms. Carbonic anhydrase seemed to have no perceptible influence on the rate of film formation. We conclude that the presence of carbonic anhydrase in either active or inactivate form did not initiate precipitation in the metastable calcifying solution.

Our findings do not provide an explanation of the results reported by McConnell *et al.* Perhaps a clearer picture of the mechanism of precipitation will emerge when a more detailed account of their experimental conditions is available.

The negative results of our attempts to obtain precipitates in vitro from metastable physiological calcifying solutions under the influence of carbonic anhydrase, together with the knowledge of the rapidity of the uncatalyzed CO_2 hydration, argue against the function of carbonic anhydrase as the "principal biochemical catalyst in vivo" for the precipitation of apatite. Moreover, our many experiments on the precipitation of calcium phosphates in the presence of carbonate (*9*) demonstrate that the carbonate ion interferes with or even inhibits apatite precipitation rather than initiates it (*10*).

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Bachra and Trautz state: "On theoretical grounds it is unlikely that the catalytic action of the enzyme carbonic anhydrase would be required for the precipitation of apatite in vitro." Our report (*1*) states quite clearly (in connection with the experiments in which we used synthetic calcifying solutions): "First it was discovered that when the solutions were saturated with carbon dioxide by bubbling the gas through them, the *enzyme was not required* in order for a deposit to form" (italics added).

They say, "It is unlikely that carbonate (or bicarbonate) ions are essential for the precipitation of apatite in vitro or in vivo." Inasmuch as the carbonate hydroxyapatite (bone mineral) which forms in vivo *universally* contains (*2*) a small percentage of carbon dioxide (as carbonate), it makes no sense whatever to assume that it can form in the absence of carbonate (or bicarbonate) ions. This statement is true regardless of whether or not a precursor of bone mineral is involved.

Since our original results appeared (*1*), we have reduced the period of time required to obtain the precipitations from several days to several hours. The fact that Bachra and Trautz obtain different results does not appear to be justification for their publicly questioning our results, particularly in view of the fact that they did not investigate the same systems. Our systems were open either to laboratory air or to carbon dioxide at atmospheric pressure (as clearly indicated), whereas theirs were in erlenmeyer flasks which were "closed with paraffinized cork stoppers."

If, as they imply, our depositions were caused by bacterial or fungal growth, we found no visible evidence of it. Application of their speculation to our later experiments (those requiring merely 6 to 8 hours) would certainly require modification of existing theories concerning the proliferation of bacteria (or fungi) in environments unfavorable to their growth. Under any circumstances the production of carbonic anhydrase by microorganisms cannot be eliminated as a principal cause of the precipitations which Bachra and Trautz obtained.

Furthermore, Bachra and Trautz choose to ignore the fact that sulfanilamide was found to function as a specific inhibitor of carbonic anhydrase in our experiments, both in those involving saliva and in those involving synthetic calcifying solutions. The presence of carbonic anhydrase in bovine submaxillary gland extract has recently been demonstrated (*3*). Earlier experiments (*4*) had demonstrated that this extract was capable of initiating deposition of synthetic calculus.

To be sure, the hydration of carbon dioxide by carbonic anhydrase is virtually instantaneous, but this fact is completely irrelevant to our conclusion that carbonate (or bicarbonate) ions must be present during the formation of bone mineral. It is evident that carbonate ions must be available during the mineralization process because a carbonate-containing substance cannot be formed in the absence of carbonate ions. As we previously clearly stated: "However, when these synthetic solutions were not initially saturated with carbon dioxide and when the apparatus was enclosed in an atmosphere of carbon dioxide, the results . . . were essentially similar to those obtained with boiled saliva: that is, no significant deposit was obtained unless carbonic anhydrase was added, and addition of sulfanilamide prevented formation of a deposit."

We note that the report of Bachra and Trautz contains one principal point: they were unable to duplicate our results, and they state that our experimental conditions "were not specified in sufficient detail." There is indeed a limitation on the length of reports in *Science*, but it might have been helpful had Bachra and Trautz read with comprehension some of the statements that we did make. Thus, they could have avoided several illogical interpretations, and they surely could have obtained further information (without resorting to publication of their dubious speculations) if, indeed, they were not present when additional details were supplied at a meeting of the International Association for Dental Research, held from 23 to 26 March 1961 in Boston (*5*).

In summary, it should be quite apparent that their experiments do not present any evidence which vitiates either our experimental results or the conclusions which we obtained.

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Protective Effects of Human Milk in Experimental Staphylococcus Infection

Abstract. Mice were given human milk and sublethal doses of virulent *Staphylococcus aureus* subcutaneously on 7 to 14 consecutive days. When mice of this group and mice of control groups were later challenged through intraperitoneal injection of a lethal dose of the same *Staphylococcus aureus*, the death rate for the experimental group was found to be much lower than the death rate for the controls.

During the last few decades, the progressive decline in breast-feeding in technically highly developed countries has been stimulated by cultural-anthropological factors and abetted by the apparent success of "artificial" feeding of infants. The greatest benefit in artificial feeding has been achieved by following proper aseptic rules. In technically underdeveloped countries, poor hygienic conditions make breast-feeding practically essential if young infants are to survive.

The additional claim that breast-fed infants have increased resistance to disease has been often advanced, even in the recent past (*1*), but this claim has not been supported with unequivocal scientific evidence. Clinical observations on the beneficial effect of human milk in the treatment of chronic staphylococcal infection in the preantibiotic era (*2*) belong possibly in the same category.

In the studies reported here, the synergistic action of human milk and