to decomposition. Heating at temperatures below 100° and at reduced pressures proved time consuming and inadequate. Above 80° prolonged heating always resulted in the loss of some fluorine due to hydrolysis.

Successful partial dehydration was accomplished in an evacuated desiccator over phosphorus pentoxide at 65° in approximately 50 hr. Loss of total sample weight and fluorine and phosphorus analyses indicated the hemihydrate formation. When held for 9 days at these conditions, there was no additional loss in weight and analytical data indicated pure hemihydrate.

There was no particular consistency in the rate of dehydration and influencing factors were sample size, exposed surface, condition of the phosphorus pentoxide and degree of evacuation of the desiccator.

The only evidence for the existence of a stable monohydrate was obtained by hydrating the hemihydrate. When placed at 20° in a constant relative humidity of 15% (approximately 3.5 mm. of water vapor) the hemihydrate gained sufficient weight to convert theoretically to the monohydrate.

There was no additional gain in weight with time, and there was no loss in weight when placed over anhydrous calcium chloride. The monohydrate gained in weight to the dihydrate value when placed in a 51% relative humidity atmosphere at 25°. However, this second water molecule could now be removed by drying over anhydrous calcium chloride at room temperature whereas the original dihydrate water could not be removed in this manner.

Elevated temperatures failed to yield the anhydrous salt. Decomposition with loss of fluorine resulted in every case. The theoretical composition of possible hydrates of calcium monofluorophosphate are given in Table II for comparison.

TABLE II

COMPOSITION OF POSSIBLE HYDRATES

Hydrate	Ca, %	F, %	P, %
$CaPO_3F \cdot 2H_2O$	23.0	10.9	17.8
$CaPO_3F \cdot H_2O$	25.6	12.2	19.9
$CaPO_3F \cdot {}^1/_2H_2O$	27.2	12.9	21.1
$CaPO_3F$	29.0	13.8	22.5

TABLE III

SOLUBILITY OF $CaPO_3F \cdot 2H_2O$ IN WATER

Temp., °C.	G. of $CaPO_3F$/100 ml. soln.
5	0.486 ± 0.006
17	.476 ± .006
27	.417 ± .006
37	.390 ± .006
48	.438 ± .006
58	.486 ± .006

The solubility of the salt in water (Table III) was determined by volumetric analysis of the saturated solution and all solubilities were less than 0.5 g. of $CaPO_3F$ per 100 ml. of saturated solution. Equilibrium was reached by constant stirring of a solution containing excess solid for 24 hr. with longer periods of stirring time showing no increase in solubility. After 2 months of solid–liquid contact, the salt was found to be negligibly soluble at room temperature in all organic solvents tested. These included: 95% ethanol, carbon tetrachloride, chloroform, 1,4-dioxane, tetrahydrofuran, ethyl acetate, furfural, pyridine, thiophene, ethylene glycol, ethylene glycol monoethyl ether and carbon disulfide.

Crystallographic studies were hampered by the difficulty of obtaining large perfect crystals. However, studies revealed the dihydrate crystals had an inclined extinction angle whose measurements varied between 33 and 37° and also a parallel extinction angle. These indicated the monoclinic crystal system for the dihydrate, which was also observed to form twin crystals. The hemihydrate crystals appeared light brown in color and had the same general shape as the dihydrate. However, it was suspected that they did not belong to the same crystal system but that a case of pseudomorphism had been observed.

It has been noted that the properties of monofluorophosphate compounds closely resemble those of the corresponding sulfates. This investigation shows a marked similarity between calcium monofluorophosphate and gypsum, $CaSO_4 \cdot 2H_2O$.

NORMAN, OKLAHOMA

[CONTRIBUTION FROM THE DIVISION OF PHARMACOLOGY, DEPARTMENT OF RADIATION BIOLOGY, SCHOOL OF MEDICINE AND DENTISTRY, UNIVERSITY OF ROCHESTER]

The Surface Chemistry of Bone. IX. Carbonate:Phosphate Exchange[1]

BY W. F. NEUMAN, T. Y. TORIBARA AND B. J. MULRYAN

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Under carefully controlled conditions, hydroxyapatite crystals were equilibrated with bicarbonate buffers. Bicarbonate was found to penetrate the hydration shells of the crystals and, in addition, displace phosphate ions from the surfaces of the crystals. These two physicochemical processes may account for the large amounts of CO_2 found in bone. The exchange reaction is quantitatively of the most physiological importance since the crystals of mature bone are poorly hydrated *in vivo*.

In 1881, Hoppe-Seyler[2] attempted to describe bone salt by a formulation which included carbonate ions as part of the "molecule." Today, it is generally recognized that bone salt cannot be represented as a "molecule" but rather is best described as microcrystalline material exhibiting the lattice structure of hydroxyapatite.[3] The exact nature of the ever-present carbonate of bone mineral is still uncertain, however, and there exists in the literature an array of suggestions and theories, none of which has been experimentally established beyond reasonable doubt.[3b]

None of these suggestions takes into account the recently-discovered fact that crystals of hydroxyapatite, in an aqueous medium, possess hydration shells.[4] Therefore, a series of investigations of a model system (aqueous buffer–hydroxyapatite crystals) was conducted to clarify the problem of carbonate fixation in bone.

Materials and Methods.—A well-characterized preparation of crystalline hydroxyapatite[5-8] was used for all equilibration studies. All other chemicals were commercially available, C.P. grade. The methods and apparatus used

(1) This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

(2) F. Hoppe-Seyler, *Z. Physiol. Chem.*, Berlin (1881).

(3) (a) S. Eisenberger, A. Lehrman and W. D. Turner, *Chem. Revs.*, **26**, 257 (1940); (b) W. F. Neuman and M. W. Neuman, *ibid.*, **53**, 1 (1953).

(4) W. F. Neuman, T. Y. Toribara and B. J. Mulryan, THIS JOURNAL, **75**, 4239 (1953).

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(6) J. H. Weikel, Jr., W. F. Neuman and I. Feldman, THIS JOURNAL, **76**, 5202 (1954).

(7) G. J. Levinskas and W. F. Neuman, *J. Phys. Chem.*, **59**, 164 (1955).

(8) W. R. Stoll and W. F. Neuman, THIS JOURNAL, **78**, 1585 (1956).

for equilibration have been previously described.[6] Phosphorus was analyzed by the Fiske and SubbaRow method[9]; calcium by either the Versene[10] or flame photometric methods[11]; sodium by flame photometry[8]; and CO_2 was analyzed gravimetrically after the gas had been released by acid and collected in ascarite tubes.

Results

Time Required for Attainment of Steady State.—Two experiments were performed to determine the time required for apatite crystals to equilibrate with a carbonate-containing buffer solution. In the first experiment, 2 g. of apatite crystals was added to 100 ml. of an equal mixture of 1 *M* $KHCO_3$ and 1 *M* KCl which had been adjusted to *p*H 7.5 by bubbling 100% CO_2 through the solution. After varying time intervals of rapid stirring, the crystal suspension was centrifuged, the resultant crystalline sludge transferred to special centrifugation cups[4] and centrifuged at 10,000 × *g* for 2 hr. The hydrated crystals, freed of mechanically-held water,[4,8] were then analyzed for CO_2 content. The averaged results of duplicate experiments are given in Fig. 1A.

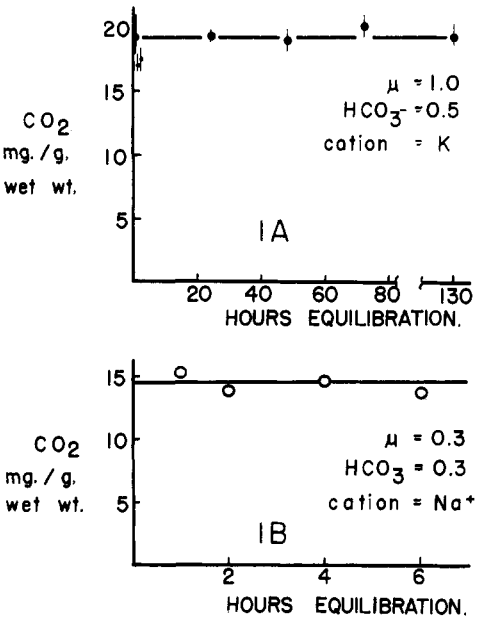

Fig. 1.—Data showing the rapidity with which CO_2-uptake by the solid phase attains steady-state conditions.

In the second experiment, conditions were altered slightly in that 4.5 g. of apatite was equilibrated with 600 ml. of 0.3 *M* $NaHCO_3$ at *p*H 7.4. The hydrated crystals were isolated and analyzed as before. These results are given in Fig. 1B.

While no claim is made that equilibrium was achieved, it is perfectly clear that the crystals' uptake of CO_2 was very fast and remained at a constant level, within experimental error, from 5 minutes (actually it required <2 hr. to isolate the hydrated crystals) to 130 hr. equilibration. Neither the nature of the cation nor the ionic strength of the buffer had any measurable effect on the time required. Accordingly, an equilibration time of 2 to 6 hr. was adopted for all subsequent studies.

Effects of Varying Bicarbonate Concentration.—Two experiments were conducted testing the effects of varying the concentration of bicarbonate ion in the solution equilibrating with the crystals. In the first case experiment I [HCO_3^-] was varied by dissolving different quantities of $NaHCO_3$; therefore, the ionic strength varied proportionately to the [HCO_3^-]. In the second case, the ionic strength was maintained at unity by mixing KCl and $KHCO_3$ in varying proportions. Within experimental error, the CO_2-fixation by the solid was uninfluenced by changes in ionic strength. In both cases, the CO_2 in the solid phase was roughly proportional to the concentration of bicarbonate in the solution phase. In neither case was the relationship linear. These results are given in Fig. 2. The legend accompanying the figure presents pertinent experimental details.

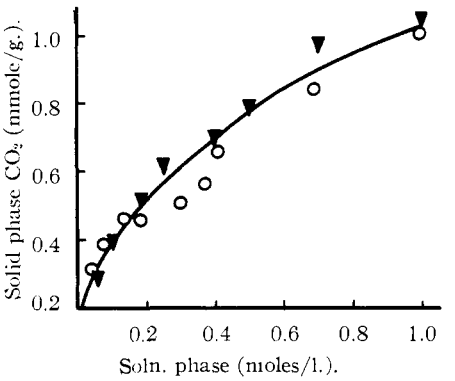

Fig. 2.—A curve showing the relation between CO_2 taken up by the solid phase and the concentration of bicarbonate in the solution. The solid triangles represent data from experiment I employing the following experimental conditions: μ = [HCO_3], cation = Na^+, *p*H 7.4, 4.5 g. apatite/600 ml. buffer, T = 25°, 2 hr. equilibration; the open circles represent data from experiment II under the following conditions: μ = 1.0, cation = K^+, *p*H 7.4, 4.5 g. apatite/600 buffer, T = 25°, 2 hr. equilibration.

In experiment I, the extent of hydration of the crystals was determined by methods previously published.[4] These data are given in Fig. 3 and demonstrate that, at high levels of CO_2 impregnation, there was a significant loss of hydration water. A simple calculation shows clearly that only a part of the CO_2 found in the solid phase can be attributed to bicarbonate in the hydration shell[12]: 0.67 ml./g. × 1 *M* HCO_3 = 0.67 mmole CO_2/g., while observed fixation was 1.05 mmole CO_2/g. This suggests that bicarbonate (or carbonate) ions entered into the crystal surface in addition to penetrating the hydration shell. In support of this, it was found in experiment II that CO_2 fixation by the solid was accompanied by a displacement of phosphate from the crystals to the solution as seen in Fig. 4.

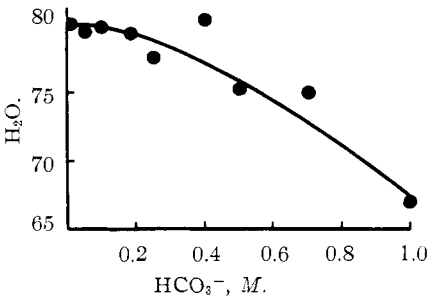

Fig. 3.—Data showing loss of hydration shell water with increasing bicarbonate concentration. Hydration water has been expressed as: wt. H_2O × 100/wt. dry apatite.

Effect of Calcium Concentration.—It has been reported[6] that the amount of readily exchangeable phosphate (as measured with radiophosphate) associated with hydroxyapatite crystals can be markedly increased by increasing the concentration of calcium in the equilibration fluid. Accordingly, an experiment was performed testing whether calcium exerted a similar influence on carbonate fixation. The results are given in Fig. 5, the legend of which contains

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(12) This calculation assumes that γ_{HCO_3} in the hydration shell equals γ_{HCO_3} in the bulk solution. If it is assumed that $\gamma_{HCO_3} = \gamma_{Cl}$ in the hydration shell, the predicted value is 0.51 m*M* CO_2/g.

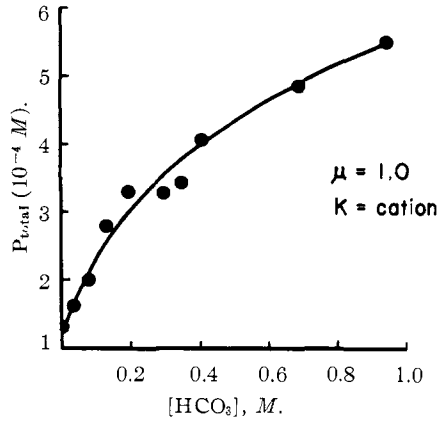

Fig. 4.—A curve showing the displacement of phosphate from the crystals to solution by increasing carbonate impregnation.

experimental details. Like exchangeable phosphate, the solid phase CO_2 content was increased by increasing calcium concentration.

The mechanism of the calcium-effect is, of course, unknown. If it is assumed that the net positive charge on the crystals is governed by the $[Ca^{++}]/[H_3{}^+O]$ ratio[6,7] of the solution, it is only reasonable that the number of surface-associated anions would increase with increasing $[Ca^{++}]$ at constant *p*H. It is of interest to calculate the CO_2 content of bone as predicted from these investigations *in vitro*.

With the following assumptions: (a) $[Ca^{++}]$, *p*H and HCO_3^- in the interstitial fluid are the same as in normal serum, namely, 1.5×10^{-4} *M*, 7.4, and 2.5×10^{-2} *M* respectively; (b) the surface area of the bone crystals is approximately 200 m.²/g.,[4,8] that of the synthetic apatite used in these studies is 68 m.²/g.[5]; (c) the extent of hydration of crystals of mature bone is 0.4 g. H_2O/g.[4,8]; one may calculate from the data in Fig. 5 thusly

$$CO_3^= \text{ in surface} = (0.395 - 0.205) \text{ mmole/g.} \times \frac{200}{68} = 0.56 \text{ mmole/g. ash}$$
(obsd.) − (initial)

$$[HCO_3] \text{ in hydration shell} = 0.025\ M \times 0.0004 \text{ l./g.} = 0.01 \text{ mmole/g. ash}$$

$$\text{Total } CO_2 = 0.57 \text{ mmole/g. or } 2.5\% \ CO_2$$

This is in fair agreement with, but lower than, the usual percentage observed *in vivo*, 4 to 5%.[3b] However, these same assumptions led to a predicted sodium content of bone of 0.2 mmole/g. ash.[8] This, too, is somewhat low, since values of 0.3 mmole/g. ash for rat bone are commonly encountered in our laboratory. It suggests the effective surface area of bone crystals may be greater than assumed, approaching 300 m.²/g.! In any event, within the uncertainties of our knowledge of the dimensions of the crystals and of the composition of the interstitial fluid of bone, the exchange:hydration shell concept can account reasonably well for the CO_2 content observed in bone.

Reversibility.—Both entry of bicarbonate ions into the hydration shell by diffusion and entry into the solid surface by exchange displacement should be readily reversible processes. An attempt was made, therefore, to demonstrate the reversibility of the system.

To 600 ml. of solution (μ = 1.0 $[K^+]$ = 1.0 *M*, $[Cl^-]$ = 0.5 *M* $[HCO_3^-]$ = 0.5 *M*, *p*H 7.4 by adjustment with gaseous CO_2) was added 10 g. of hydroxyapatite crystals. After 2 hr. or 24 hr. impregnation, the hydrated crystals were isolated by centrifugation as previously described. The hydrated crystals (5 g.) were then suspended in potassium phosphate buffer, either 0.4 or 0.01 *M*, for 2 or 24 hr. during which time CO_2-free nitrogen was bubbled to sweep away gaseous CO_2. The *p*H was either 6.0 or 5.0. In no case was the CO_2 content of the solid phase reduced to a level below that originally found present as a contaminant in the crystals, 4.7 mg. CO_2/g. wet weight. However, all of the CO_2 added to the solid by exposure to carbonate buffer was readily removed by all desorption procedures. These data are summarized in Table I.

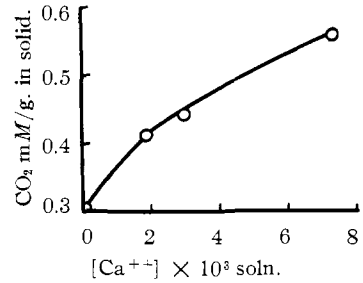

Fig. 5.—A curve showing increasing carbonate uptake with increasing calcium concentration in solution. This phenomenon cannot be ascribed to the limited solubility of $CaCO_3$ because only at the highest calcium concentration was the K_{sp} approached. Experimental conditions were: μ = 0.16, cation = K^+, $[Cl^-]$ = 0.135, $[HCO_3^-]$ = 0.025, *T* = 25°, 4.5 g. apatite/600 ml.; $[Ca^{++}]$ was varied by pre-equilibrating the apatite with small additions of $CaCl_2$ to the KCl solution *prior* to addition of bicarbonate as $KHCO_3$.

A Study of the "Hydroxyl Displacement" Proposal.—Romo[13] prepared a so-called "carbonato apatite" by mixing $CaCO_3$ with aqueous KH_2PO_4 and NaOH. The precipitate which formed was washed and then ignited at 300° for 2 hr. The infrared absorption spectrum of this solid was compared with that of an *unignited* commercial hydroxyapatite specimen. A band at 3.00 μ, present in the hydroxyapatite, was missing in the "carbonato apatite" while a band at 11.5 μ, present in the carbonato apatite, was missing in the hydroxyapatite. Absorption at 3.00 μ was attributed to hydroxyl ion absorption; that at 11.5 μ to carbonate leading Romo to conclude that carbonate displaces hydroxyl ion in the lattice.[13]

The absorption at 3.00 is *not* due to hydroxyl ion, however. Rather it is covalent hydroxyl such as in alcohols or water that absorbs at 3.00 μ. It appeared possible, therefore, that Romo's unignited control sample of hydroxyapatite merely contained adventitious water. A number of hydroxyapatite preparations were examined before and after ignition at 300° for 2 hr. In every case, a significant absorption at 3.00 μ was observed before heating but the band disappeared or nearly so after heating.

TABLE I

THE REVERSIBILITY OF THE CARBONATE PHOSPHATE EXCHANGE[a]

Impregnation		Desorption *p*H 6.0, 2 hr.	Desorption *p*H 6.0, 24 hr.	Desorption *p*H 5.0, 2 hr.
None	4.7	6.5		
	(1)	(1)		
2 hr.	19.2	5.6	5.7	(4.6)
	(6)	(6)	(2)	1
24 hr.	23.4	5.0	4.6	
	(1)	(1)	(1)	

[a] Impregnation was accomplished by exposing 10 g. of apatite to 600 ml. of buffer: μ = 1.0, cation = K^+, $[Cl^-]$ = 0.5, $[HCO_3^-]$ = 0.5, *p*H 7.4, *T* = 25°, time indicated. Desorption was accomplished by placing 5 g. of isolated impregnated crystals in 600 ml. of phosphate buffer, 0.4 or 0.1 *M*, at the indicated *p*H and for the indicated time. *p*H adjustment was made with KOH. Numbers in parentheses indicate the number of individual samples averaged.

In dried specimens, studies in the infrared have shown CO_2 to be present as carbonate to the exclusion of bicarbonate.[14,15] If $CO_3^=$ substituted for OH^-, the maximum content of CO_2 theoretically possible according to Romo's proposal is 4.4%. Yet, two preparations made according to

(13) L. A. Romo, THIS JOURNAL, **76**, 3924 (1954).

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(15) A. S. Posner and G. Duyckaerts, *Experientia*, **10**, 424 (1954).

Romo's procedure gave solids which, on analysis, contained 12.8 and 17.4% CO_2.

It must be concluded that Romo's "carbonato apatite" was actually a variable mixture of $CaCO_3$ and CO_2-containing apatite. His infrared evidence must be disregarded because he failed to heat his control specimen of apatite.

Adsorption of Gaseous CO_2.—Because bone specimens do not lose CO_2 on standing in air, it seems reasonable that inappreciable quantities of gaseous CO_2 are adsorbed *in vivo*. Nonetheless, as a precautionary check, apatite samples were equilibrated at room temperature with a "physiological" atmosphere containing 5% CO_2 for 66 hr. and analyzed for total CO_2 content immediately. The values obtained, 0.88%, agreed with control samples, 0.84%, within experimental error.

Discussion

In view of the many suggestions concerning the nature of the carbonate in bone mineral it is pertinent to focus on those for which reasonable experimental support exists and to refute those which have no factual foundation.

Klement's suggestion[16] that the CO_2 of bone mineral represents "entrapped" alkali bicarbonates can be dismissed without further consideration. In these experiments, no precipitation occurred, therefore no entrapment was possible, yet CO_2 entered the solid phase at all concentrations of HCO_3^- in the buffer solutions. Besides, if mechanical "entrapment" were occurring during bone formation *in vivo*, the most likely anion to be "trapped" would be chloride ($Cl^- \simeq 0.135$ *M vs.* $HCO_3^- \simeq 0.025$ *M*) yet the chloride content of bone mineral is always very low.[17]

Romo's recent contention[13] that carbonate displaces hydroxyl ions in the lattice is actually a restatement of an old and discarded idea. The X-ray diffraction evidence and structural considerations rule out this concept.[3b,18] In addition, the limited evidence brought forth by Romo was artifactual as shown by data given above.

From time to time, there have been suggestions that the CO_2 of bone may be present as a separate phase of calcium carbonate, presumably calcite. Two kinds of evidence refute this idea. First, in the present studies, the solid phase incorporated CO_2 at all concentrations of [HCO_3] though the K_{sp} of $CaCO_3$[19] was never exceeded. Earlier workers have obtained similar results.[20] Second, attempts to demonstrate the presence of $CaCO_3$ by X-ray diffraction have failed[21] despite the adequate sensitivity of the method. It might be argued that $CaCO_3$ is present only as a monolayer on the crystal surfaces and, because such a monolayer would be different structurally from the surface of a calcite crystal, the K_{sp} of $CaCO_3$ does not apply. Such a "modified" concept in the final analysis, is perilously close to a postulate of surface adsorption.

"Adsorption" is a relatively old explanation of the presence of carbonate in hydroxyapatite preparations and also of the observed variability in the Ca/P ratio.[2] The small size and large surface area of the crystals made this postulate seem reasonable. Yet, how can *ions* adsorb to a surface? Electroneutrality requires that either (a) a neutral compound be adsorbed or (b) some like-charged ion be displaced from the surface.

For such an ion-for-ion displacement the term, "ion exchange" seems preferable to "adsorption." Indeed, essentially all of the surface-chemistry of hydroxyapatite can be formulated on an ion-exchange concept.[3b] All of the results of the present studies are consistent with the view that the mechanism of CO_2 fixation involves a displacement of phosphate groups by carbonate groups in the surface and an equilibration of the crystal hydration shells with bicarbonate ions in the bulk solution. Because the crystals in mature bone are poorly hydrated,[4] however, most of the CO_2 present *in vivo* (*vide supra*) must be in the form of surface-held carbonate. The ionic environment of these carbonate groups must be quite similar to that of carbonate in calcite because infrared absorption spectra of the carbonate grouping are the same for both bone and calcite.[14,15] The *in vitro* data are insufficiently precise to determine whether the carbonate–phosphate exchange occurs on an equivalent or equimolar basis. However, at low levels of CO_2 impregnation the presence of bicarbonate in the buffer elevates the concentrations of *both* calcium and phosphate dissolved at steady state conditions. This suggests the exchange may be equimolar, thusly

$$2HCO_3^- + \text{solid} \longrightarrow \text{solid} + 2HPO_4^{=} + Ca^{++}$$

Such an equimolar ion exchange process should be readily reversible and in the present experiments it was.

In animals,[22] in glycol-ashed specimens of bone[23] and probably[24] in geological specimens the carbonate does not appear to be entirely available and does not wholly equilibrate with labeled carbon dioxide or show preferential dissolution in acid. It is necessary to postulate, therefore, that in the process of maturation and crystal growth either (a) the surfaces become "entrapped"[24] as suggested by Hendricks and Hill or (b) the surface carbonate groups become incorporated in the lattice itself.[25]

The present chemical evidence does not differentiate between these two possibilities, but it is pertinent to note that sodium ion which enters the crystal surface by ion exchange for calcium[8] and which no one suggests as an internal lattice constituent, also becomes "unavailable" *in vivo*.

ROCHESTER, NEW YORK

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(20) M. A. Logan, *Physiol. Rev.*, **20**, 522 (1940).

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