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The Surface Chemistry of Bone. VII. The Hydration Shell[1]

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It is shown that considerable amounts of water are intimately associated with the crystals of hydroxyl apatite and of bone. Three kinds of studies demonstrated: (a) that this water is only partially removed by high speed centrifugation; (b) that this hydration shell does not contain the electrolytes of the bulk solution; and (c) the crystals adsorb water in accordance with the Brunauer, Emmett and Teller theory. Adult, compact shaft contains little, if any, water that can be removed by centrifugation and there is insufficient water present to furnish complete hydration shells for the mineral crystals.

The crystals of bone mineral and its synthetic counterpart, hydroxyl apatite, are extremely minute[2]; therefore, these materials evidence a tremendous specific surface, actual measurements varying from 70–200 m.^{2}/g.[3,4] The significance of this enormous surface has been emphasized recently and the suggestion has been made that the exchangeable ions of bone mineral reside in these surfaces.[4b]

Work on other systems possessing exchangeable ions such as clays and synthetic exchange resins has demonstrated a consistent correlation between the type and number of exchangeable ions present and the tendency of the solid to adsorb water vapor.[5] The purpose of the present investigation was to demonstrate the occurrence of a hydration shell on the crystals of both mineral and synthetic apatite. The term hydration shell should not be inferred to indicate the mechanism by which the water is held by the crystals.

Experimental

Centrifugation Studies.—The first attempt to demonstrate the existence of a hydration shell involved the use of high-speed centrifugation. It was assumed that at least some of the hydration shell would withstand a reasonable centrifugal force, whereas mechanically held water would be removed. In fact, just such a procedure has been used in the preparation of hydrates for analysis.[6]

Three grams of synthetic hydroxyl apatite was stirred rapidly with one liter of dilute potassium phosphate buffer (1×10^{-4} *M*, *p*H 7.4) for 26 hours. This dilute slurry was then transferred to a fine, sintered-glass filter and, with the aid of gentle suction, all excess solution drawn off. The remaining wet powder was subjected to low speed centrifugation (approx. 800 × *g*) for 10 minutes and then transferred to a covered weighing vessel. Four samples (approx. 300 mg.) were weighed out and dried at 115° to constant weight. Four additional samples were weighed out to special centrifuge cups (Fig. 1) and centrifuged at high speed for one hour in a Servall vacuum centrifuge (Model SS-1). The samples were weighed again and, finally, dried at 115° to constant weight. From these data the per cent. water retained by the apatite following high-speed centrifugation could be calculated. The entire process was performed four times to give a series of values at forces varying from 10,000 to 40,000 × *g*.

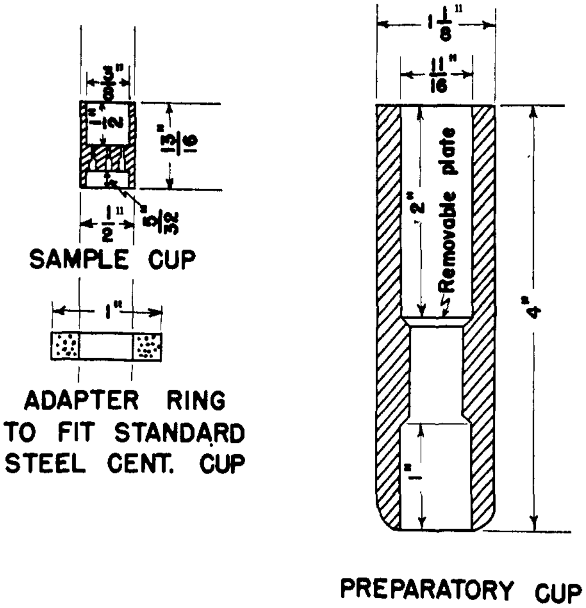

Fig. 1.—Specially designed cups for the removal of all but the hydration shell from bone salt preparations. A small pad of glass wool is used to support the crystals.

A second series was conducted under identical conditions except that the apatite was stirred with 1 liter of unbuffered $CaCl_2$ (10^{-4} *M*, *p*H 7.4).

The results of these two series were comparable and are presented in Fig. 2. There was a marked drop in the

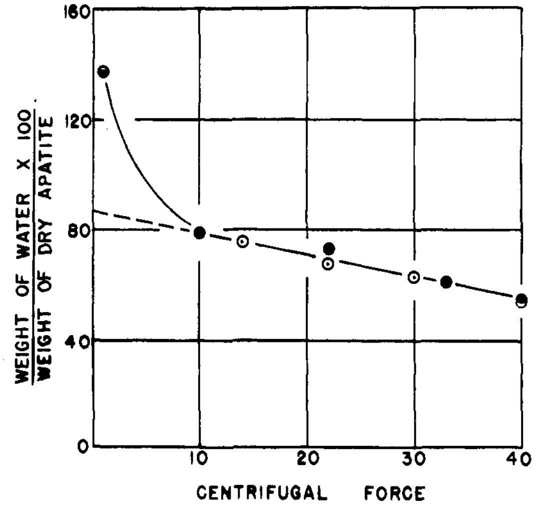

Fig. 2.—Data showing the water retained by synthetic apatite preparation at varying centrifugal speeds. Dry weight was obtained by heating overnight at 110°.

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[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] R. A. Robinson, "Metabolic Interrelations," 3rd Josiah Macy Conf., 1951, p. 271.

[3] M. Falkenheim, W. F. Neuman and H. C. Hodge, *J. Biol. Chem.*, **169**, 713 (1947).

[4] (a) S. B. Hendricks and W. L. Hill, *Proc. Natl. Acad. Sci.*, **36**, 731 (1950); (b) W. F. Neuman, "Metabolic Interrelations," 2nd Josiah Macy Conf., 1950, p. 32.

[5] A. G. Keenan, R. W. Mooney and L. A. Wood, *J. Phys. Colloid Chem.*, **55**, 1462 (1951).

[6] H. H. Willard and T. Y. Toribara, THIS JOURNAL, **64**, 1760 (1942).

amount of water held by the crystals as the force of centrifugation was increased to 10,000 × *g*.; thereafter the water content decreased slowly but linearly with increasing force of centrifugation.

The simplest explanation of the curve is that mechanically held water was removed with a separating force below 10,000 × *g*; at higher rates of centrifugation the water loss was due to removal of water from the outermost portions of the hydration shell.

Radiophosphorus Studies.—This explanation could be easily tested by placing the crystals in a radiophosphate buffer and then centrifuging. As mechanically held water was removed, it would take its radiophosphate with it. Water of the hydration shell should contain no radiophosphate capable of removal.

Five grams of synthetic hydroxyl apatite crystals was stirred for 48 hours in 600 ml. of solution containing 84.5 g. of Na_2HPO_4 adjusted to *p*H 7 with the aid of a small amount of HCl. One ml. of radiophosphate solution (approx. 2 mc.) was added, stirring continued for two hours and, as before, the crystals were collected on a sintered glass filter, centrifuged at low speed (800 × *g*) for 10 minutes, weighed into the special centrifuge cups and centrifuged at high speed, one group of samples at 17,000 and one group at 43,000 × *g*. The specific activity (SA) of the apatite crystals in each centrifugation group was determined by dissolving in 2% HCl and counting aliquots under a conventional, thin, mica-window Geiger–Müller tube. These data are plotted as points in Fig. 3, and confirm the existence of a hydration shell.

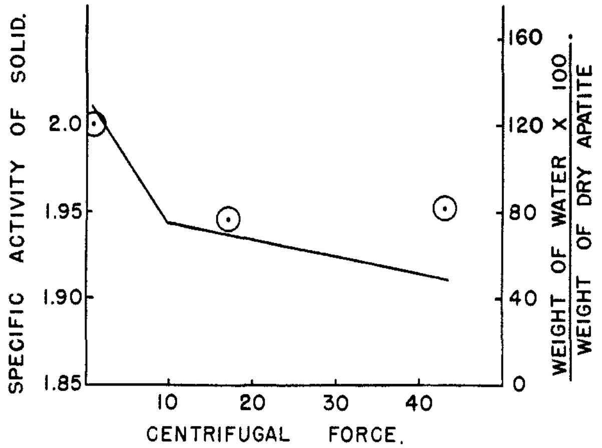

Fig. 3.—Data showing that the water removed at high speed centrifugation does not contain bulk electrolyte. The water content (solid line) decreased with increasing centrifugal force while the specific activity (open circles) of the dried solid did not. The size of the circles indicates the standard error of the specific activity measurements. Dry weight was obtained by heating overnight at 110°.

Vapor Pressure Studies.—As an independent check on the results obtained by centrifugation, attention was turned to experiments involving the equilibration of dried, synthetic apatite crystals with various pressures of water vapor. For these experiments, an apparatus very similar to that described by Wiig and Juhola[7] was employed. Precautions and technique were taken from Keenan, Mooney and Wood.[6]

Great difficulty was encountered in removing the last traces of adsorbed gases from the apatite. This was overcome by the following procedure.

Samples were dried overnight at 110° in order to remove all traces of the hydration shell. The sample bulb was pumped out at 10^{-3} mm. pressure for 30 minutes to obtain the dry weight of the sample. Pretreatment consisted of letting water vapor in until the manometer registered a pressure more than desired and then pumping the sample out for 20–30 minutes. This procedure was repeated and water vapor was admitted until the desired pressure was obtained. The routine was followed for each point on the "adsorption" curve. The "desorption" curve was obtained by pumping out the sample for suitable periods of time. All measurements were made at 25 ± 0.05°.

After time for the system to reach equilibrium (4 hours or more), the vapor pressure of the water vapor over the apatite was read on an oil manometer by means of a cathetometer. The sample bulb was then sealed off from the system by means of a stopcock, removed and weighed on a semimicro balance to determine the amount of H_2O adsorbed.

The apatite preparation proved to be a powerful adsorbent of water vapor. A typical adsorption isotherm curve is given in Fig. 4.

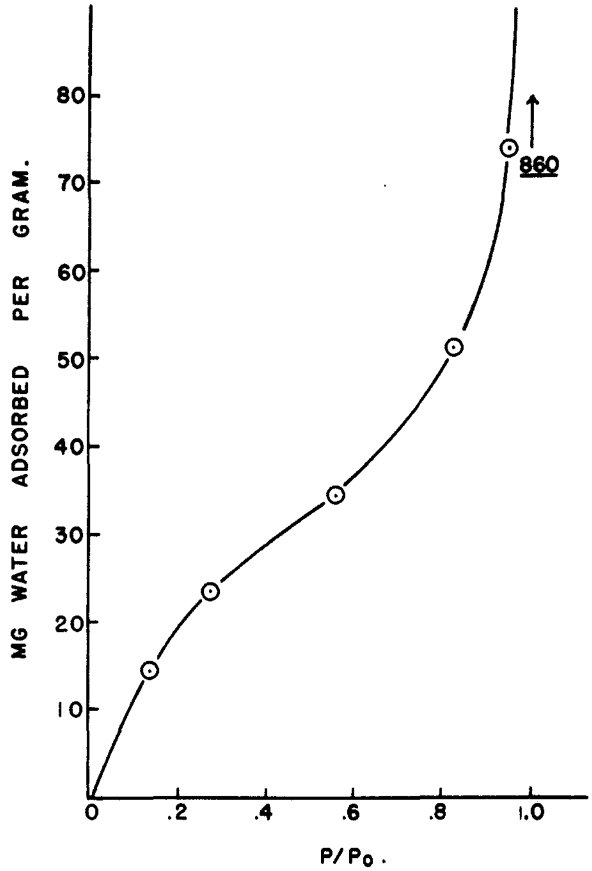

Fig. 4.—An adsorption isotherm showing the relation between vapor pressure and the water adsorbed by synthetic apatite. P_0 represents the vapor pressure of pure water at the temperature of the experiment. The value 860 at $P/P_0 = 1$ was obtained from extrapolation of data given in Fig. 2. This value must be considered very approximate because of difficulties in controlling temperature during centrifugation.

Keenan, Mooney and Wood obtained similar results in studying kaolinite, a specially purified clay exhibiting ionic-exchange properties. They successfully applied BET theory[8,9] in the analysis of their data. This theory permits a translation of the adsorption isotherm into linear form, as in Fig. 5, giving a ready evaluation of the parameter, V_m, the number of molecules required to cover the surface with a monolayer. However, in a system containing exchangeable ions capable of hydration V_m may not represent a monolayer in the ordinary sense. In the words of Keenan, *et al.*,[6] V_m cannot—"be used for measurement of surface area, but it still represents a perfectly well-defined quantity characteristic of the given system. In the present application, owing to the presence of exchangeable ions with their energy of hydration, the surface is certainly not uni-

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(7) E. O. Wiig and A. J. Juhola, This Journal, **71**, 561 (1949).

(8) S. Brunauer, P. H. Emmett and E. Teller, *ibid.*, **60**, 309 (1938).

(9) S. Brunauer, L. S. Deming, W. E. Deming and E. Teller, *ibid.*, **62**, 1723 (1940).

form and it may be that the BET theory is giving an averaging effect for two sets of adsorbed molecules with large differences in their mean energies."

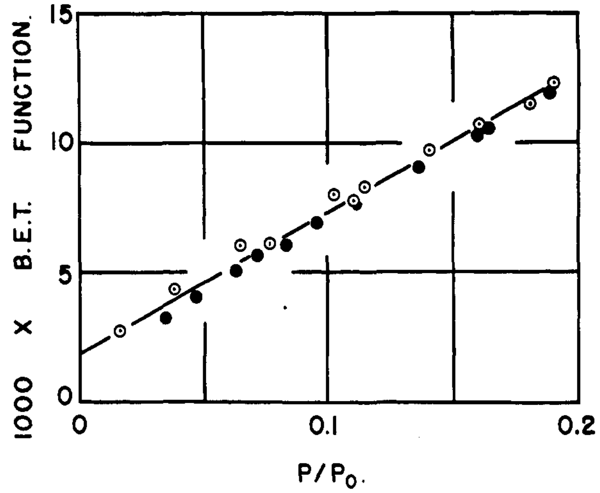

Fig. 5.—The application of BET theory to the adsorption of water by synthetic apatite. Open circles represent points obtained during adsorption. Solid circles represent points obtained during desorption.

In spite of difficulties in interpretation it is interesting to compare the results obtained in the present study of apatite with the data reported for the calcium form of kaolinite. This comparison is given in Table I. Actually the values for V_m correspond fairly well to monolayer dimensions if compared with the surface areas given by the nitrogen method.

TABLE I

A COMPARISON OF WATER ADSORPTION BY APATITE AND KAOLINITE

Material	Nitrogen surface area, m.²/g.	Water "Monolayer" (V_m), mg./g.	Exchangeable calcium, mg./g.	Molecules of water per ion
Kaolinite[5]	27.6	8.06	0.043	8.5
Synthetic Apatite	67.8	17.6	.037	...

Studies with Fresh Bone.—Having established a hydration shell on crystals of synthetic apatite, it was of interest to obtain comparable results on a specimen of fresh bone. Therefore, the centrifugation experiments reported above were repeated using unashed, fat-extracted, powdered veal bone.[10] These data are given in Fig. 6.

While evidence was obtained for the occurrence of a hydration shell, the striking observation was that the fresh bone showed only 40% as much hydration as the synthetic apatite. This could not be attributed to difference in crystal size or to the quantities of exchangeable ions present. Data given in Table II prove the fresh bone sample to have a smaller crystal size! In addition, any hydration of the protein present would be expected to introduce a positive error since the results have been presented in terms of per cent. water per gram of dry ash.

TABLE II

A COMPARISON OF DATA ON CRYSTAL SIZE OF BONE AND APATITE[a]

Preparation	Surface area, m.²/g.	Exchangeable phosphorus, %
Synthetic apatite	67.8	4.2
Glycol ash veal bone	117	13

[a] R. A. Robinson, from electron microphotographs, estimated that the crystals of the synthetic preparation were roughly "twice the size" of the glycol ash bone crystals. The size estimates correspond fairly well with surface area measurements.[11]

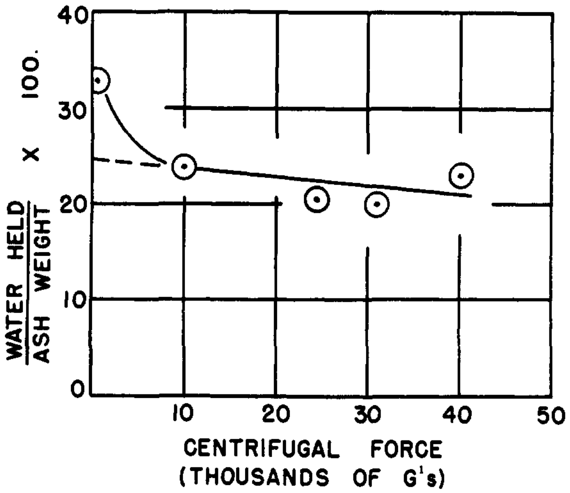

Fig. 6.—Data showing the water retained by fresh de-fatted veal bone preparation at varying speeds of centrifugation.

Because of the important differences between apatite and unashed bone and because of the uncertainties introduced by fat-extraction procedures, an attempt was made to study the water distribution in untreated bone.

Shafts of an aged cows' metacarpals were freed of most muscle, sawed into two-inch sections, split, cleaned of marrow and frozen. The time from slaughter to freezing was kept to less than four hours. While frozen, all bits of superficial muscle and connective tissue and, in addition, several mm. of bone were removed from the bone sections with the aid of a small motor driven rasp. The cleaned sections were cracked with a hammer and ground with bits of Dry Ice in a Wiley mill at −20° to pass a 40-mesh screen. The frozen powder was sealed in an airtight container and, after warming to room temperature, was weighed into special centrifugation cups (Fig. 1). The water content before and after centrifugation was determined by drying in a vacuum oven overnight at 60°. Equivalent results were obtained at 85°, but higher drying temperatures gave erratic results presumably due to decomposition. Such treatment has been shown to remove the hydration shell of synthetic apatite crystals.

The water lost upon centrifugation was negligible and, again, the water content of fresh bone was much less than expected if the crystals possessed complete hydration shells. The observed weight loss on drying was 11.5 ± 0.3% regardless of centrifugal force.

Discussion

The water associated with the crystals of both synthetic hydroxyl apatite and bone when in aqueous media does not appear to be due either to capillary condensation or to the formation of a crystalline hydrate. Neither mechanism would well explain (a) the linear reduction of the volume of bound water by centrifugal force nor (b) the adsorption of water vapor in conformity with BET theory. The formation of a true hydrate would necessarily alter crystal structure but X-ray diagrams kindly obtained by R. L. Griffith of Eastman Kodak Company indicated no detectable change. Since water held by the crystals did not contain the electrolytes of the bulk solution, it seems unlikely that mechanical forces are responsible. Because crystals of bone strongly adsorb anionic dyes such as alizarin, it seems more likely that the water shell is the result of boundary phenomena associated with the tiny, positively-charged crystals. Alternatively, part of the bound water

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(10) W. F. Neuman and B. J. Mulryan, *J. Biol. Chem.*, **193**, 237 (1951).

(11) R. A. Robinson, *J. Bone Joint Surg.*, **34A**, 389 (1952).

may be due to the partial hydration of individual, exchangeable ions in the crystalline surfaces. Additional data are required before final conclusions can be given.

Two important considerations arise from the work reported above. The first, purely technical, is that bone mineral can be isolated free of "contaminating" solution. The second, of even more physiological interest, is that much of the water content of fresh, unashed bone is "bound" water, water of the hydration shell.

By virtue of the enormity of its crystalline surfaces bone mineral evidences a unique ability to exchange and adsorb surface ions.[4b] To study this phenomenon it has been necessary, in most instances, to use radioisotopes. With isotopic materials it is possible to examine bone:buffer equilibria without effecting an actual separation of the bone powder. It is necessary only to analyze the solution phase before and after equilibrium has been reached. With many substances, this limitation presents great technical difficulties and in some cases no isotopic preparations are available. Now that it has been shown that powdered bone can be centrifuged free of all mechanically held solution, it is possible to isolate the bone mineral for analysis without resorting to any procedure that can alter equilibrium conditions, washing for example. To cite three pertinent cases, this new technique will facilitate the study of the binding by bone of CO_2, citrate and fluoride.

The notion that the bulk of the water in bone is not free, but is associated with the crystals poses interesting questions. Is not such bone relatively removed from the body's circulation? How can the surface ions of the crystals undergo exchange with similar ions in the circulating fluids? When bone-seeking radioisotopes gain access to the body, how can they penetrate to the crystals of the adult bone?

The answers to these questions cannot be given with certainty, but the present evidence is suggestive. It is a well established generalization that the older the bone anatomically, the lower its water content and the greater its density. Further, the comprehensive work of Amprino[12] has demonstrated that all bone, with large variations according to the part of the skeleton and to the species, undergoes continual reworking by Haversian displacement. These new Haversian systems exhibit a low density as measured by X-ray opacity[13-15] and presumably, therefore, have a higher water content.

If, as it now appears, there is little or no free water in the older and established Haversian systems, two conditions prevent the interchange of ions: (a) ions can reach the area by diffusion only since the circulation is negligible and (b) diffusion through a solid, as in this case, is an extremely slow process.[16]

This conclusion offers an explanation for the otherwise puzzling results recently reported by a number of investigators. These results fall into two categories: (a) the observations that certain constituents (CO_2, Na and H_2O) of the bone mineral, which presumably are located in the crystalline surfaces, do not enter into full equilibrium with the blood in protracted experiments with isotopes,[17-19] and (b) autoradiographs of sections of bones from *adult* animals receiving radiocalcium or radiophosphorus show areas which have not taken up isotope.[20-23]

Apart from actual accretion, the concepts of ionic exchange[24] and recrystallization of bone mineral[25] gave a partial explanation for the incorporation of isotopes by bone, but afforded no explanation for the fact that some areas of bone *did not* take up isotopes. In this connection, the correlation between areas of active growth and active isotope incorporation is now much clearer.

Why the crystals in unashed bone are unable to bind their full complement of water is not at all clear. The facts suggest that there is an intimate connection (actual bonding) between the crystals and the organic phase of bone. In support of this, are recent electron-microphotographs of intact sections of bone which show the flat surfaces of the tabular crystals contiguous with the flat surfaces of the collagen fibers.[26] Such intimate connection between collagen and crystals may explain the low water content of fresh bone and the increased availability of exchangeable ions noted on ashing.[24]

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(12) R. Amprino, *Z. Zellforschung*, **37**, 144 (1952).

(13) P. LaCroix, personal communication.

(14) R. Amprino, *Z. Zellforschung*, **37**, 240 (1952).

(15) A. Engström and R. Amprino, *Experientia*, *VI*, 267 (1950).

(16) H. E. Buckley, "Crystal Growth," John Wiley and Sons, Inc., New York, N. Y., 1951, p. 258.

(17) H. L. Kornberg, unpublished results.

(18) D. L. Buchanan and A. Nakao, *Federation Proc.*, **11**, 19 (1952).

(19) I. S. Edelman, A. H. James and F. D. Moore, *ibid.*, **11**, 40 (1952).

(20) J. S. Arnold, *ibid.*, **11**, 5 (1952).

(21) C. L. Comar, W. E. Latz and G. A. Boyd, *Am. J. Anat.*, **90**, 113 (1952).

(22) C. P. Leblond, G. W. Williamson, C. F. Bélanger and J. Robichon, *ibid.*, **86**, 289 (1950).

(23) R. Amprino, *Experientia*, *VIII*, 20 (1952).

(24) H. C. Hodge, "Metabolic Interrelations," 1st Josiah Macy Conf., 1949, p. 49.

(25) W. F. Neuman and B. J. Mulryan, *J. Biol. Chem.*, **185**, 705 (1950).

(26) R. A. Robinson and M. L. Watson, *Anat. Record.*, **114**, 383 (1952).