NATIONAL BUREAU OF STANDARDS REPORT

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Progress Report

on

Blood: Bone Equilibrium in Calcium Homeostasis

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U.S. DEPARTMENT OF COMMERCE

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THE NATIONAL BUREAU OF STANDARDS

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Blood: Bone Equilibrium in Calcium Homeostasis

By

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Abstract

Data on the solubility of bone in vitro are plotted so that the slope of the line through the experimental points yields the Ca/P ratio of the equilibrating salt. The slopes of the lines for child and calf bone corresponded best to the ratio 8/6, that of octacalcium phosphate, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$; the slope for adult bone approximated that of hydroxyapatite, $Ca_{10}(OH)(PO_4)_6$.

This is the first direct evidence that octacalcium phosphate is present in bone. Its presence in "young" bone, as contrasted to "adult" bone, is taken as further evidence for the participation of octacalcium phosphate as a precursor in the formation of the apatitic crystallites in vivo. The presence of octacalcium phosphate in bone introduces a new concept to be considered in relation to the chemistry of growth, healing, and diseases of bone, and it helps to explain factors that control composition of serum.

The nature of the dynamic equilibrium between the mineral component of human bone and its ions in solution in tissue fluid is unknown. A few years ago the theory advanced by MacLean and Urist (1) was generally accepted, and experimental studies on the mode of action of parathyroid hormone (e.g. Talmage et al (2), Copp (3)) were designed in terms of it. Their hypothesis held that normal tissue fluids were 'supersaturated' with respect to whole bone; that the bone mineral maintained only 'hypoparathyroid levels' of calcium and phosphorus by physical processes and that a parathyroid controlled 'vital activity' caused destruction of whole bone, release of mineral and increase in the concentration of calcium in the tissue fluids. Maintenance of a calcium concentration gradient therefore not only required continual secretion of parathormone but also continual osteoclastic resorption of whole bone.

In 1957, Nordin (4) suggested that the situation be re-examined in terms of solubility theory and showed that calf bone powder would maintain reproducible levels of Ca and P in vitro. Later, MacGregor and Nordin extended the work and showed that in both trihydroxymethylaminomethane: HCl (5) and carbonic acid: potassium bicarbonate buffer systems (6) at ion strengths of 0.15 and at 20°C, human dead bone powder would maintain the levels of Ca and P found in normal tissue fluids if the pH at the solid: solution interface was about 7.0 to 7.1 (6). This pH is intermediate between the intracellular pH of 6.8 and the extracellular pH of 7.4, and the concept of a pH gradient across the blood:bone barrier is not unattractive.

The equilibration studies showed that the ion-concentration product, $[Ca^{++}]^3 [PO_4]^2$, expressed as a negative logarithm (pK) was relatively constant over the pH range 6.4 to 7.6 and ranged from 26.0 to 26.8 and was not related to pH. On the other hand, the ion-concentration product $[Ca^{++}][HPO_4]$, correlated highly with pH (5). On this evidence, MacGregor and Nordin postulated that, on purely empirical grounds, the cubed-squared product best represented the physico-chemical relation between normal human bone and its ions in 'equilibrium' with it. It was pointed out that no theoretical significance could be attached to the tricalcium phosphate product on this basis and that the authors did not necessarily consider that this salt was the relevant solid phase of 'the exchangeable calcium pool'.

The chemical nature of the micro-crystals in bone and the physical properties of its surfaces have been the subject of discussion for many years. The conventional view (7) is that hydroxyapatite (HA) is the relevant compound. The basic model is modified in detail by internal lattice faults with entrapped contaminants, and the free surfaces are believed to have suffered heteroionic exhange with ions such as Sr^{++}, Mg^{++}, Na^+, P^-, $CO_3^=$, and citrate. In consequence, quantitative analysis of whole bone has yielded no definitive data.

In view of the possibility that one or more sparingly soluble calcium phosphates $\{Ca_5OH(PO_4)_3, Ca_8H_2(PO_4)_6 \cdot 5H_2O, \beta-Ca_3(PO_4)_2, CaHPO_4, CaHPO_4 \cdot 2H_2O\}$ could be present in bone, assumptions regarding the stoichiometry of the equilibrium between the solid phase and the aqueous solution are undesirable. It is easily shown (8,9,10) that solubility data can be treated in a way so that such assumptions become unnecessary. The formula for any calcium phosphate may be written in generalised form as $Ca_xH_y(PO_4)zH_2O$, where $2x + y = 3$ and y may have negative values (e.g. for HA, x = 5/3 y = -1/3 and z = 1/3). The condition for equilibrium between an aqueous solution and the generalised salt required that there be no change in free energy for the reaction:

1

$$x \, Ca(OH)_2 + H_3PO_4 + (z - 2x) \, H_2O = Ca_xH_y(PO_4) \cdot zH_2O$$
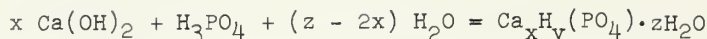
That is, the sum of the chemical potentials of the components given on the left side of the equation is equal to the standard free energy of formation of the salt on the right-hand side.

$$x \, \mu_{Ca(OH)_2} + \mu_{H_3(PO_4)} + (z - 2x) \, \mu_{H_2O} = \Delta F^\circ_{xz}$$

If we then assume that the chemical potential of the water is essentially constant for dilute solutions and that the chemical potentials of $Ca(OH)_2$ and H_3PO_4 may be expressed as usual in terms of their ionic activities, then:

$$\mu_{Ca(OH)_2} = \mu^\circ_{Ca(OH)_2} + RT \ln (Ca^{++})(OH^-)^2$$

$$\mu_{H_3PO_4} = \mu^\circ_{H_3PO_4} + RT \ln (H^+)^3(PO_4^{\equiv})$$

and we can derive the result:

$$\log (Ca^{++})(OH^-)^2 = -\frac{1}{x} \log (H^+)^3(PO_4^{\equiv}) + K_{xz}$$

where the parentheses represent ionic activities and K_{xz} is a constant specific for the applicable salt.

Therefore if negative log $(Ca^{++})(OH^-)^2$ is plotted against negative log $(H^+)^3(PO_4^{\equiv})$ for a series of solutions in equilibrium with the above salt, the points should fall on a straight line with a slope of $-\frac{1}{x}$ where x is the Ca/P molar ratio in the salt. In other words, the slope is dictated by the stoichiometry of the reaction. The position of the line is determined by the value of K_{xz} which cannot be calculated without assumptions concerning the appropriate activity coefficients. However, in a series of experiments at constant ionic strength it is usually assumed that the activity coefficients are also constant, and therefore the slope of the line is valid even though its position is made somewhat obscure by the replacement of Ca^{++} and $PO_4^{\equiv}$ ion activities by their concentrations.

This method has been applied to the data of MacGregor and Nordin (5) and MacGregor (11). In the first case negative log $(Ca^{++})(OH^-)^2$ was plotted against negative log $(H^+)^3(PO_4^{\equiv})$ using the data from experiments in which adult dead bone powder was equilibrated with 'tris' and cacodylate buffers. Unitary activity coefficients were assumed for the calcium and phosphate ions. The graph is shown in Fig. 1. The slope of the line of best fit obtained by linear regression analysis was -0.632 which represents a Ca/P ratio for the solid phase of 9.494/6.

Secondly, the results of experiments with child dead bone powder were treated by this method and the results are shown in Fig. 2. In this case the slope was -0.743 representing a Ca/P ratio of 8.076/6.

It would appear then that in the child the solid phase in equilibrium with body fluids is octacalcium phosphate (OCP), $Ca_8H_2(PO_4)_6 \cdot 5H_2O$, while in the adult the Ca/P ratio of the equilibrating solid phase is more nearly that of HA. This interpretation is dependent on the degree of confidence we can place in the slopes of the fitted lines.

A standard correlation analysis yields a correlation coefficient, r and a regression coefficient, b. The significance of these coefficients can be decided using standard and distribution transformations. Applying this method to the human bone powder we find: -

Bone	r	Significance	b	Significance	Interpretation
Adult	-0.983	0.001	-0.632	0.001	Ca/P probably $\frac{9}{6}$ or $\frac{10}{6}$
Child	-0.976	0.001	-0.743	0.001	Ca/P probably $\frac{8}{6}$

Considering the complex form of the basic distribution of the regression coefficients, it is extremely difficult to decide whether the observed value of $-\frac{1}{x}$ i.e. the Ca:P ratio, is most likely to represent an integer ratio of 9/6 or 10/6. In practice, and in view of the relatively low S.E. of the slopes, we can say that it is unlikely that the adult bone powder has yielded a Ca/P stoichiometric ratio

2

of 8/6.

The above data indicate that the solid phase saturating the solutions in the experiments with child bone is OCP but they should not be construed to mean that bone salt in children is exclusively OCP. The behavior is also consistent with the view that the major phase is unreactive or does not have a definite solubility, while OCP - even though a minor phase - governs the apparent solubility. It has been shown in fact that the rates of dissolution and recrystallization of OCP appear to be considerably greater than those of HA (12).

Other indications support the idea that OCP may be present in bone. The 'platey' habit of crystallites in bone has been interpreted as indicating that OCP participated in their formation (12). Evidence has been presented recently to support the view that growth of HA crystals depends on a mechanism in which OCP is the initial precipitate (13, 14). The incorporation of phosphate into the apatite lattice has been shown by Dallemagne et al to involve initially an acidic phosphate which we believe is OCP (15). On the other hand, the adult bone data indicate that HA probably dominates the equilibration in aged formed bone.

Thus, the burden of evidence now available supports our considered view that bone mineral is first formed as OCP and that there are spontaneous ageing changes (hydrolysis) which convert OCP into HA. In the child, where bone growth is maximal, there tends to be more OCP in the microcrystals than in the older subject where the reorganization of the OCP to HA overtakes the precipitation process.

Since the new feature of our theory concerns the presence of OCP in 'young' bone, we have applied the calculation to the data of Nordin (4) who studied the equilibration of powdered calf bone with 0.15 M tris and cacodylate buffers at 37°C (Fig.3). His data yielded a correlation coefficient of -0.9930 which is significant at the 0.1% level and a regression coefficient of -0.7378 which is also significant at the 0.1% level. This regression coefficient represents a Ca/P stoichiometry of 8.13/6. It is highly improbable that this observed ratio could have arisen by chance if the true Ca/P ratio was 7/6 or 9/6. (P < 0.001) and confirms the dominance of the OCP stoichiometry in equilibrations with 'young' bone.

Since there appears to be a difference in the physico-chemical behaviour of young and adult bone, it may be possible to explain the raised serum inorganic phosphate concentrations in children by the greater solubility of OCP as compared to that of HA. MacGregor and Nordin were aware that their 'ion product' theory of blood:bone exchange and the role of the parathyroid glands in maintaining the level of the equilibrium (16) might not explain adequately the situation in child plasma. Possible explanations advanced by MacGregor (17) included the suggestions that child plasma might contain a larger proportion of Ca and P binding substances, that the metabolic pH gradient between the bone mineral tissue fluid interface and the circulating body fluids might be steeper due to increased metabolic activity in the young, or thirdly, that there might be a qualitative difference in child bone. This last now appears more plausible and explains the observed differences, at least in part.

Our present ability to re-interpret the child data, which to some extent conflicted with the 'solubility' hypothesis of parathyroid function, now yields substantial support to that thesis, since it allows rational explanation of the fact that plasma inorganic phosphates in young mammals are higher than in adult man, whereas the plasma calcium concentration is usually fairly constant at about 2.5 mM. In point of fact it is the calcium homeostasis which is the specific necessity for optimal cellular function.

The biological significance of these findings remains to be examined in detail. They suggest that OCP is present in greatest amounts in newly formed bone, whether child or adult, and that the chemical properties and interactions of two crystalline species must be taken into account for a full understanding of bone growth and remodelling, bone healing, and various pathological conditions.

In a sense this paper can only be a preliminary communication on the basis of retrospective analysis of equilibration data. It is now necessary to re-examine this line of inquiry. In particular, the pure salts should be subjected to the same chemical treatment as the bone powder to verify the position of the fitted lines as well as their slopes. Regardless of the uncertainty suggested by this point, the present evidence for the presence of OCP in vivo is substantial and it introduces a new factor of considerable physiological potential. Far from detracting from the concept of a metabolic process modulating a physico-chemical exchange mechanism, these data may now help to explain many of the inconsistencies previously observed with the in vitro models of skeletal behaviour.

Acknowledgement

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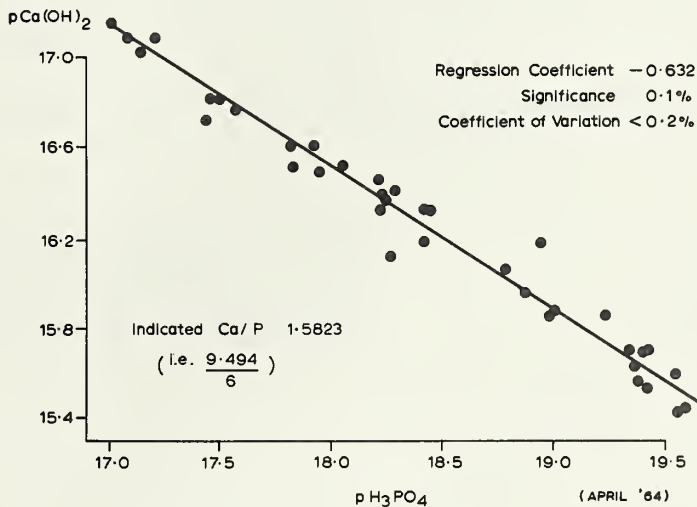17. MacGregor, J. The blood:bone equilibrium. Symposium on Bone Biodynamics (1963).

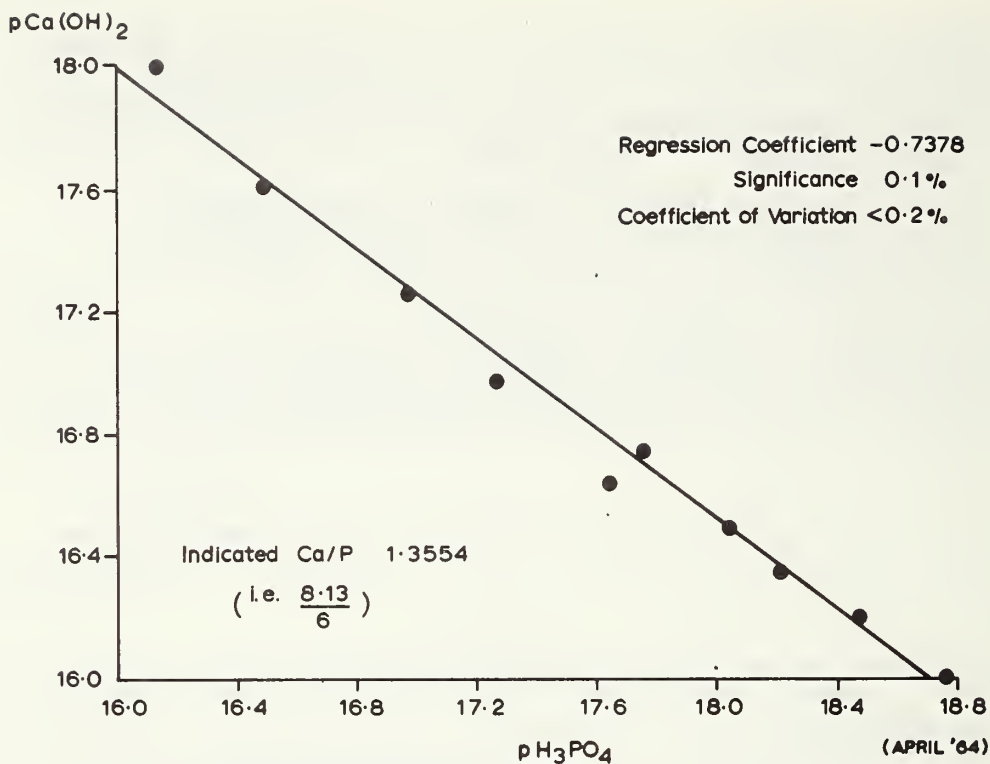Figure 1. Chemical Potential Relation in Equilibration Studies With Powdered Adult Bone

Figure 2. Chemical Potential Relation in Equilibration Studies
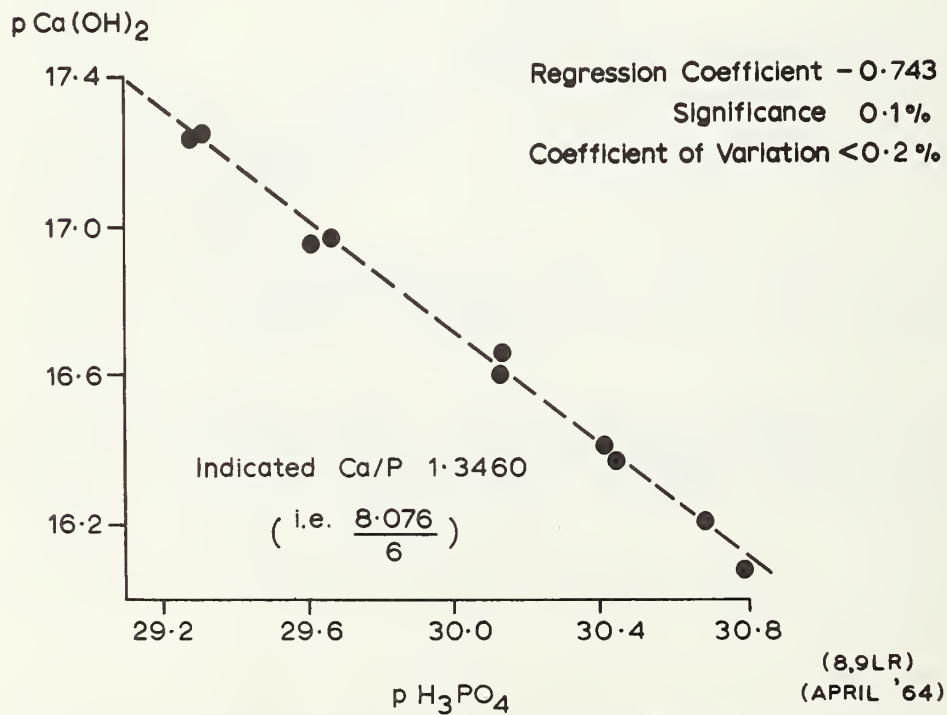With Powdered Calf Bone

Figure 3. Chemical Potential Relation in Equilibration Studies
With Powdered Child Bone