THE THEORY OF PRIMARY CALCIFICATION IN BONE

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THE theory of primary calcification was advanced in 1928 by Kramer and Shear[1] to explain the stages of mineral precipitation in bone in course of formation. If this theory is to apply to the process of ossification submitted by Roche[2], it will refer to the beginning of the fourth stage, that of the deposit of salt in organic bone.

According to Kramer and Shear, bone salt is not tricalcium phosphate, but is, at any rate at the beginning of calcification, secondary phosphate containing calcium and phosphorus in the quantitative ratio of 1·29. These authors refuse to admit the existence of tricalcium phosphate as a chemical entity and they consider the nature of bone salt to be quite unknown; their hypothesis is that the Ca/P ratio which chemical analysis reveals in bone attains to the normal value of adult tissue (± 2·20), since dicalcium phosphate absorbs lime or carbonate in course of the calcification.

This opinion is derived from the following:

(1) If the classic formula $Ca_3(PO_4)_2$ is attributed to tricalcium phosphate, the formation of this salt must result from a reaction of the fifth order, the possibility of which is open to dispute. Shear and Kramer[3] affirm that they have never been able to obtain proof of the existence of a compound having this formula.

(2) Kramer and Shear refer to the experimental results of Holt, La Mer and Chown[4] and apply to them the solubility product law. Their opinion is that the precipitation of phospho-calcium salt takes place only at the moment when the solubility product of dicalcium phosphate $pK_{\text{sp}} = [Ca^{++}] \times [HPO_4^{=}]$ is attained in the liquid phase. This precipitate does not, therefore, correspond to the tertiary salt but to the secondary salt of the type termed by mineralogists 'brushite'.

In an attempt to verify these results obtained in the realm of physical chemistry by experiments on animals, Kramer and Shear made rats rachitic, then cured them and examined the nature of the salts freshly deposited in the osteoid tissue.

They expected to find a Ca/P ratio corresponding to that of dicalcium phosphate; but, on the contrary, they found that the calcium and the phosphorus formed a quantitative ratio greater than that of tertiary salt (1·94).

Although these results constitute a set-back to the theory of primary calcification, it has later been supported by important arguments in its favour.

The chemical study of bone callus[5,6], of the embryonic bone and tooth[7], and of bone of periostic origin[8], reveals that young osseous tissue is far richer in phosphorus as compared with calcium than the adult bone. Fig. 1 shows the evolution of the Ca/P ratio in bone of periostic origin. In tissue of recent formation its value is very low, but it increases progressively as ossification takes place; it passes 1·29, the value of secondary phosphate, and 1·94, that of tertiary phosphate; it then goes beyond this figure and, following the precipitation of calcium carbonate, halts around 2·20, the characteristic figure of adult bone.

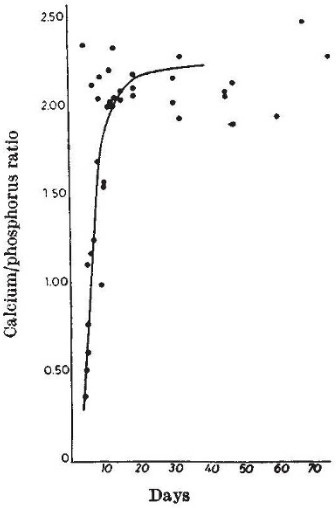

Fig. 1. RISING VALUE OF THE CALCIUM-PHOSPHORUS RATIO DURING OSSIFICATION OF THE HYPERTROPHIC PERIOSTEUM

One would gladly interpret these results by submitting that, at a certain point in the development of bone, its salt corresponds to brushite susceptible of being transformed into tricalcium phosphate following molecular changes due to local physico-chemical conditions.

The possibility of the presence of brushite in young bone is also indicated in the results of Ettori, Grangaud, Benoit and Clavert[9]. These authors study the chemical nature of bone formed in the medulla of some of the long bones following œstradiol administration to pigeons. They determine both calcium and phosphorus together in the newly formed medullary trabeculæ as in the surrounding old diaphyseal bone; they find calcium and phosphorus in a quantitative ratio often nearer to that of secondary salt than to tertiary salt; pigeons are treated from five to a hundred days with a daily injection of 0·25 mgm. of œstradiol dipropionate (the lowest Ca/P ratio would seem to appear after twenty days of treatment).

After examining the whole field connected with primary calcification, we begin to wonder if the arguments which sustain this hypothesis are very convincing.

If the smallest Ca/P ratio found by Roche and by ourselves in bone in course of formation equalled that of secondary phosphate (1·29), which is the salt of calcium and phosphorus richest in the latter element and the least soluble, there would be no doubt. How are we to explain by Kramer and Shear's theory that in very young bone the Ca/P ratio may be less than 1·29 ? We have found 0·35, and Roche has observed still lower values.

Following these considerations, we proceeded to work out the American authors' theory experimentally from its foundations.

If we add lime to phosphoric acid so as to register pH continuously, we obtain the curve (broken line) of Fig. 2. The pH rises regularly until a precipitate appears; at this point it falls temporarily. The formation of the precipitate clearly retards the alkalinization of the liquid phase by the lime. The pH begins to rise again when the concentration of the anions in solution appreciably diminishes.

At whatever point on the neutralization graph the precipitate phase appears, it always corresponds to tricalcium phosphate; this is demonstrated both chemically and physically[10].

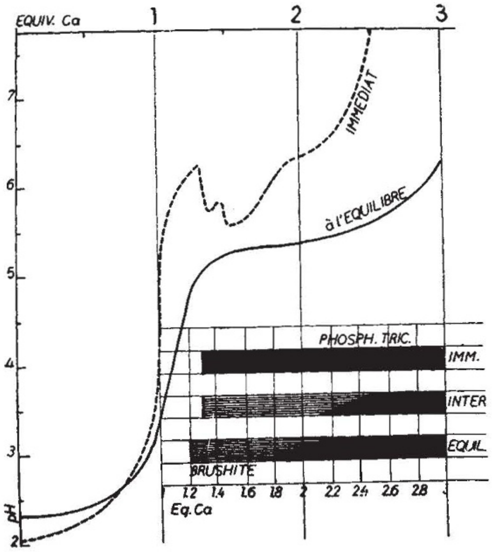

Fig. 2. TITRATION CURVES OF PHOSPHORIC ACID NEUTRALIZED BY LIME ; - - - -, DURING NEUTRALIZATION ; ——, AFTER EQUILIBRIUM. INSET, NATURE OF THE SOLID PHASE AT DIFFERENT TIMES

We are also able to fix each stage of the process of neutralization in separate preparations which we can preserve until equilibrium is obtained ; if we take *p*H readings after 15 days, we do not register the same curve : it is much more regular and it has descended in the course of establishing the equilibrium (Fig. 2, full line). This descent is due to precipitation which has slowly taken place and to modifications undergone by the solid phase ; the space between the two curves is the greater because of the abundance of the latter. The composition of the liquid phase has been modified during the establishment of equilibrium, and so has that of the solid phase ; the precipitate is no longer the same at all points of the curve.

From 1·2 to 1·7 equivalents of lime, the precipitate consists of pure brushite. From 1·7 to 2·3 equivalents it is a mixture of tricalcium phosphate and brushite, with the latter gradually diminishing as a greater quantity of lime is added to the acid. Beyond 2·3 equivalents of lime, the solid phase consists of pure tertiary salt.

In the course of establishing this equilibrium, which requires only a few weeks, the precipitate has undergone an intermediate evolution. The original tertiary salt was completely transformed into brushite from 1·3 to 2·05 equivalents of lime and partially from 2·05 to 2·50 equivalents. Brushite, therefore, within certain limits develops and then regresses. This is due to the inverse operation of two reactions : one tends to form brushite at the expense of tertiary salt and the other consists in hydrolysis of the brushite, resulting in reforming tertiary salt.

If the first precipitate formed on contact of the ions Ca^{++} and $PO_4^{\equiv}$ always corresponds to tertiary phosphate, is it possible that, in bone in course of calcification, the neutral salt originally crystallized alters to brushite and then returns to its original condition ? Such a conception in no way agrees with observations which have been made on the deposit of mineral matter in the osseous tissue during growth.

In the course of another series of experiments, we made a particular study of the liquid phase in equilibrium with the precipitate[11]. We observed that in preparations containing brushite crystals, the product $[Ca^{++}] \times [HPO_4^{=}]$ is equal to the solubility constant of secondary salt (Fig. 3, circles). On the other hand, wherever tertiary salt is pure in the solid phase, the liquid phase is under-saturated with secondary salt (Fig. 3, points), and its mineral composition obeys the law $pK_{sp} = [Ca^{++}]_3 \times [PO_4^{\equiv}]_2$. Consequently, in a certain zone of the neutralization graph, although tertiary salt is present, the ionic product corresponds to the solubility constant of secondary salt because the solid phase contains brushite. Nevertheless, in certain samples, there is so little of the latter, on well-defined portions of Fig. 2, that chemical analysis fails to reveal it and returns a Ca/P ratio corresponding to tertiary salt. It can be observed optically only. Kramer and Shear, who do not make use of the optical method of research and of characterization of crystals, have admitted that the total of the precipitate corresponds to dicalcium phosphate with the addition of lime and carbonate ; although it contains only very occasional crystals drowned in the mass of neutral salt ; but these crystals suffice to condition the saturation of the liquid phase with respect to dicalcium phosphate. Further, the zone of the neutralization graph in which the liquid phase is under-saturated with secondary salt and the precipitate entirely constituted of tertiary salt and containing no brushite has not been examined by Kramer and Shear.

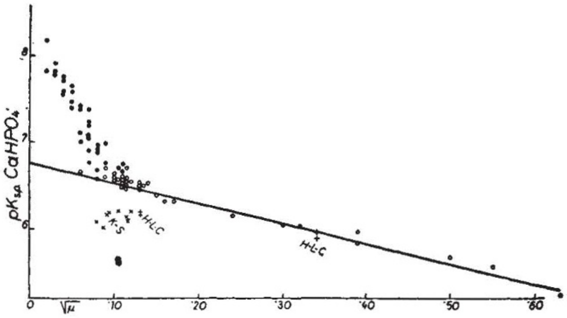

Fig. 3. EFFECT OF THE IONIC STRENGTH OF THE LIQUID PHASE ON THE SOLUBILITY CONSTANT OF DICALCIUM PHOSPHATE

○, Solid phase contains brushite crystals ; ●, solid phase consists of tertiary salt only ; *H-L-C*, values obtained by Holt, La Mer and Chown ; *K-S*, values obtained by Kugelmass and Shohl.

In spite of these authors' observations, the existence of tertiary salt must be admitted as a possibility.

We have also worked out Ettori, Grangaud, Benoit and Clavert's experiments, choosing the condition which enabled them to obtain the lowest Ca/P ratios in pigeon's bone. Unlike Ettori, we prefer, for greater precision, to separate the newly formed bone from the old bone and determine the calcium and phosphorus that both contain. We have never found the Ca/P ratio to be less than that of tricalcium phosphate. So as to confirm the absence of brushite in folliculinic bone by a more exact process than chemical analysis, we measure the refractive index of old bone cortex and of new medullary bone, freed from their organic matter by prolonged boiling in glycerine containing 6 per cent potash.

		Refractive index of mineralized bone (λ = 589·1 A.)
Pigeons control	cortical bone	1·595
	old medullary bone	1·595
Pigeons treated	cortical bone	1·591 ± 0·0004
	new medullary bone	1·590 ± 0·0018
Cattle	cortical bone	1·590
α-Tricalcium phosphate		1·590

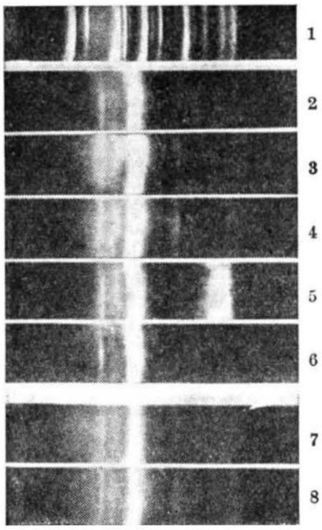

Fig. 4 X-RAY PHOTOGRAPHS OF DICALCIUM PHOSPHATE (1), OF FOLLICULINIC BONE (2–6), AND OF NORMAL DIAPHYSEAL PIGEON BONE (7–8)

As is shown in the accompanying table, the refractive index of new and of old mineralized bone exactly corresponds to that of α-tricalcium phosphate, the hydrated form of tertiary salt[12].

In collaboration with H. Brasseur, we submitted old bone and folliculinic bone to X-ray diffraction, and we obtained in every instance the radiogram of α-tricalcium phosphate, which is very different from that of dicalcium phosphate[13].

These radiograms are grouped together in Fig. 4.

There remains only to consider the opinion of Kramer and Shear in respect to tricalcium phosphate.

We are enabled to characterize tricalcium phosphate in its two forms, hydrated and non-hydrated, by the use of X-ray diffraction and by precise chemical research in collaboration with H. Brasseur[14]. The principal salt present in bone, isomorphous to apatite, is constituted of three molecules of $Ca_3(PO_4)_2$ combined with two molecules of water. When heated to 700° C., the latter are eliminated and tricalcium phosphate answers to the classic formula $Ca_3(PO_4)_2$. Heated to 900°, with a stoichiometrically defined amount of calcium chloride, of fluoride, of carbonate or of oxide, it combines with the latter to form a true apatite.

There is therefore no longer any doubt as to the nature of the stable chemical composition of tricalcium phosphate.

Greenwald[15] introduced an important element which might, if supported by new experimental proofs, cause a modification in the arrangement of the atoms of calcium and of phosphorus in the formula of tricalcium phosphate and which would furnish an answer to Kramer and Shear's objection relative to the manner of formation of tertiary salt. Greenwald submits that tricalcium phosphate is a salt of the acid $HCaPO_4$, the existence of which he thinks he has observed in solution, both in the ionized and non-ionized form. Thus, tricalcium phosphate might originate in course of a second-order reaction which agrees perfectly with the manner of its experimental formation. In its hydrated form, then, α-tricalcium phosphate would correspond to the formula $[Ca(CaPO_4)_2]_3.H_2(OH)_2$.

Thus the theory of primary calcification, having lost both its experimental and theoretical foundations, fails to explain the mechanism of calcification.

How then can we explain, apart from the theory of primary calcification, the progressive rise of the Ca/P ratio in bone in course of formation ?

It is clear that the ions $PO_4^{\equiv}$, when freed from phosphoric esters in the course of the second phase of ossification, must attach themselves to an organic element, for at this point we do not find a sufficient quantity of mineral cations in the bone to constitute an insoluble compound with phosphorus. Possibly it may be to the pre-osseous organic substance itself that they become attached ? But we can say with certainty that by whatever means the $PO_4^{\equiv}$ ions may be fixed in the precursor of the bone ossein, the Ca/P ratio of bone in formation continually rises because the microcrystals of α-tricalcium phosphate are progressively deposited in a tissue rich in $PO_4^{\equiv}$ ions. It is but a coincidence that the Ca/P ratio touches the value 1·29 and in no way explains the presence of brushite in bone in course of formation.

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THE WEST AFRICAN CACAO RESEARCH INSTITUTE

By O. J. VOELCKER

A STATEMENT made by the Secretary of State for the Colonies in the House of Commons in 1946 disclosed the fact that a sum of £1¼ million was to be set aside from the profits of the West African Cocoa Control Scheme for cacao research in West Africa. This decision gave effect to the proposal put forward by the previous Government[1].

That a sum of this magnitude should be devoted to research on one specific crop is a matter of considerable interest and importance in the history of agricultural research in the British Colonial Empire. The reason for research on this scale is not far to seek. The territories of the Gold Coast and Nigeria normally produce for export about half the world's 600,000 tons annual consumption of cacao beans, and the wealth of the Gold Coast, and to a lesser degree that of Nigeria, is intimately bound up with the production of cacao. Within recent years, pests, diseases and the degradation of certain soils have led to marked reduction in crops.

The genus *Theobroma* is not indigenous to Africa. The introduction of *Th. cacao* to West Africa is generally considered to have taken place in the