LI. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

A REPLY TO SHIPLEY, KRAMER AND HOWLAND.

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IN a paper which appears in the present number of this *Journal*, Shipley, Kramer and Howland [1926] give an account of experiments in which calcification was found to occur in slices of cartilage and bones of rachitic animals when these were kept *in vitro* in normal serum or in aqueous solutions containing calcium salts and inorganic phosphates.

Arguing from these very interesting results they reject the hypothesis [Robison, 1923] that calcification depends on the activity of an enzyme secreted by the osteoblasts and hypertrophic cartilage cells. A final decision on this question cannot be arrived at without further investigation, but I wish to deal very briefly with some of their remarks which appear to be based on misapprehension with regard to the experimental evidence on which the hypothesis rests.

The brief description of the theory on p. 385 does not correctly represent my statements. Although tertiary calcium phosphate (or a more basic salt) is deposited from the tissue fluids, the amount of this salt existing in solution in these fluids and in blood constitutes an exceedingly small fraction of the total inorganic phosphate, most of which occurs as HPO_4'' and $\text{H}_2\text{PO}_4'$ ions.

On p. 384 the following statement appears: "Robison obtained uniformly negative results with concentrations of calcium and phosphorus corresponding to those found in normal serum and also negative results with higher concentrations *when no esterase was added*." *No esterase was added* in any one of these experiments, the whole purpose of which was to demonstrate the pre-existence and the precise location of the enzyme in the tissue itself. It is quite true that the concentration of calcium in the solutions of calcium glycerophosphate was much higher than it is in blood, but these solutions contained no inorganic phosphate at all. It was not claimed that the conditions represented those found *in vivo* or that the results were exact histological pictures of normal calcification. But it was claimed that a bone when supplied with calcium and with a suitable phosphoric ester can, by its own enzymic activity, hydrolyse this ester and deposit the inorganic phosphate thus produced in the form of calcium phosphate, and further that this deposition takes place in precisely those regions in which normal calcification would have occurred.

The criticism on p. 386 that "the illustrations resemble the diffuse deposition of calcium phosphate from very alkaline solutions" misses the obvious fact that without the action of the enzyme no inorganic phosphate existed and therefore none could be deposited. Their suggestion that the deposit may have been due to faulty staining may be passed over without comment.

Again on p. 386 the remark "there seems to be no special necessity...to postulate the necessity of an enzyme" indicates some misapprehension of the facts. The presence of the enzyme in bone and in ossifying cartilage has not been postulated but experimentally proved and its complete absence from cartilage in the early stages of bone development, prior to the beginning of ossification, has also been proved [Martland and Robison, 1924, 2].

Page 385. The presence of calcium hexosemonophosphate in the blood, however interesting from other aspects, is not in any way necessary to the enzyme theory of calcification. As I have clearly pointed out, the requirements of the scheme would be satisfied equally well by glycerophosphate or any other phosphoric ester which is hydrolysed by the bone enzyme and not by the muscle esterase. The presence of such an ester has been definitely proved [Kay and Robison, 1924] although its chemical nature has not yet been established. It is unnecessary to discuss here the question of its relationship with hexosemonophosphate, but with regard to the footnote on p. 385 it should be noted that the reducing power of hexosephosphoric esters is not the same as that of the free hexose but is always much lower [Robison, 1922].

In the next paragraphs there appears to be some confusion of ideas with regard to esterases. The authors state that when serum is allowed to stand in contact with its clot the conversion of organically bound phosphorus can occur "even in the absence of esterase." The increase of inorganic phosphate which occurs under such conditions has been recognised by many workers, and has been shown [Martland and Robison, 1924, 1; Martland, Hansmann and Robison, 1924; Martland, 1925] to be due to the action of a phosphoric esterase present in the corpuscles. This enzyme is quite distinct from that which occurs in bone. Its optimum p_H lies at or slightly below 7.0 and it hydrolyses those phosphoric esters of the blood upon which the bone enzyme has no action. The converse also holds. *The phosphoric esters of the blood are of more than one type and may serve more than one purpose.*

The occurrence of the enzyme in rachitic bones (p. 385) presents no difficulty, for a number of other factors besides the enzyme are necessarily involved in calcification and failure of any one of those would be enough to prevent the normal process from taking place [*v.* Robison and Soames, 1925]. It is also not difficult to understand the presence of the enzyme in the intestinal mucosa since it may serve for the hydrolysis of phosphoric esters present in foodstuffs.

Its function in the kidney has been the subject of recent investigation [Eichholtz, Robison and Brull, 1925] and evidence has been obtained that

the enzyme may play an important part in the normal secretion of inorganic phosphates.

The liberation of inorganic phosphate by the action of an esterase in any tissue will not necessarily be followed by the deposition of calcium phosphate in that tissue. Other factors such as the p_H of the tissue fluids may play a determining part.

The results obtained by Shipley, Kramer and Howland give a certain reasonableness to their argument that the intervention of a phosphoric ester and esterase is unnecessary in order that calcification should take place, but they are in no way opposed to the views which I have put forward, for it has not been suggested that the enzymic hydrolysis of the phosphoric ester is the sole mechanism in calcification. A possible supplementary mechanism has even been suggested [Robison and Soames, 1924], viz. that the osteoblasts and hypertrophic cartilage cells may have the power to raise the p_H of the tissue fluids in which they are bathed. The effect of this on a solution saturated with respect to $\text{Ca}_3(\text{PO}_4)_2$ and CaCO_3 would be to cause a precipitation of both these salts. This would account for the presence of calcium carbonate which forms 10% of the inorganic constituents of bone, and, according to Bassett [1917], is precipitated separately and not as part of a complex carbonato-phosphate. At the same time the activity of the enzyme would be increased, thus greatly accelerating the precipitation of calcium phosphate over that of the carbonate. It is not necessary to assume that the p_H is raised to 8.4. A small upward change would produce an effect of the sort described. The results obtained by Shipley, Kramer and Howland could be satisfactorily explained on this assumption although they provide no proof of its correctness. It is not unreasonable to suppose that this mechanism would be destroyed by treatment with chloroform, cyanide, formalin, etc.

Unless it is accepted that the presence of enzymes in body tissues may be entirely fortuitous and have no significance whatever it is idle to argue that they cannot function in any particular way because their optimum p_H is impossible of attainment by the cell fluid. It would be more reasonable to enquire whether their optimum p_H (as determined *in vitro* with tissue extracts) may not be modified within the body¹. One fact bearing on this question may be given. The optimum p_H of the enzyme in kidney extracts is the same as that in bone extracts but definite evidence of marked enzymic activity was obtained when surviving kidneys were perfused with blood at the normal p_H *whatever may have been the p_H of fluid within the kidney cells* [Eichholtz, Robison and Brull, 1925].

In the foregoing discussion, the results of Shipley, Kramer and Howland, in so far as they relate to the calcification of cartilage immersed in solutions of calcium salts and inorganic phosphates, have been accepted without

¹ The influence of endocrine secretions on the optimum p_H of liver diastase has been studied by Langfeldt [1921] who obtained evidence of positive effects with adrenaline and thyroiodine. Similar investigations on the bone enzyme by Martland and Robison and by Demuth [1925] have so far failed to detect any such influence.

question. It is obvious that the results obtained with normal serum fit in equally well either with their views or with my own, since the necessary phosphoric ester might be present in such serum. Their statement that in the experiments with aqueous solutions calcification occurred best "when the concentration of the bone-forming constituents and the reactions are nearly those of normal serum" requires further consideration.

It is probable that only about 53 % of the calcium in serum is diffusible through normal animal membranes [Cameron and Moorhouse, 1925] and that the percentage of ionised calcium¹ is still lower than this², whereas in aqueous solutions of calcium phosphate containing 10 mg. calcium per 100 cc. upwards of 80 % of the calcium is present in the form of its ions. It is the ionic concentration and not that of total Ca (and P) which determines the condition of supersaturation and eventual precipitation of solid salt, and in respect of calcium ions the solutions used by Shipley, Kramer and Howland were much more concentrated than normal serum. These solutions were supersaturated with respect to tertiary calcium phosphate and the results obtained appear to have depended largely on this condition of supersaturation, for the authors state "calcification occurs best when no precipitation whatever takes place for the obvious reason that precipitation lowers the concentration of calcium and phosphorus."

It is very suggestive also that in these experiments calcification was delayed or totally inhibited by the addition of 1 or 2 % of albumin to the solutions, a result which does not accord very well with the view that the factors determining the process in these aqueous solutions were exactly the same as those which operate *in vivo*. The effect of the protein can be readily understood if it is assumed that a sparingly dissociated calcium-protein compound is formed, since the concentration of calcium ions and the degree of supersaturation would thereby be reduced. The existence of such a compound probably accounts for at least part of the non-diffusible calcium in plasma.

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¹ Or the "activity coefficient" in the terminology of the modern theories of strong electrolytes.

² Neuhausen and Marshall [1922] from electrometric determinations conclude that 80 ± 5 % of the calcium in serum is present in some un-ionised form.