XXI. A CHEMICAL STUDY OF DEFECTIVE OSSIFICATION IN RACHITIC ANIMALS.

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ANY attempt to understand the mechanism of ossification leads naturally to a consideration of those conditions which give rise to abnormalities in bone development. Apart from their intrinsic interest the investigation of such cases may throw light on the process as it takes place in the normal individual and it was for such reasons that the present study of ossification in rachitic animals was commenced.

Among the very large number of published papers on the cause and nature of rickets, relatively few deal with the chemical aspects of the defective ossification, the immediate cause of the failure to deposit the normal amount of calcium salts in the rachitic bone. Some fairly complete analyses of the inorganic constituents of normal and rachitic bones have been published, and the concentration of calcium and phosphate in the blood has been studied by a number of investigators. While their evidence shows that in children and in animals, rickets is frequently associated with a lowered content of inorganic phosphate in the blood, this is not always the case and is not specific of the condition [Hess and Unger, 1922]. There is little evidence of any significant variation in the concentration of total calcium [Howland and Kramer, 1921]. It would seem that the investigation must go deeper if this complex chemical problem is to be solved.

On the basis of the suggestions put forward in previous papers [Robison, 1923; Robison and Soames, 1924¹] the deposition of calcium phosphate in the growing bone would be adversely affected by any of the following factors:

¹ *Correction.* In our previous paper [Robison and Soames, 1924] the equation (1) on p. 752 (and the same equation on p. 749) should read

$$[\text{Ca}^{++}]^3 \times [\text{PO}_4^{=}]^2 = K_1 [\text{Ca}_3(\text{PO}_4)_2].$$

The limiting value of $[\text{H}^+]$ below which $\text{Ca}_3(\text{PO}_4)_2$ would be deposited (p. 752) is $K_2 \sqrt{\frac{k_2^3}{k_1[\text{HPO}_4^-]}}$.

The conclusion that this value depends on the respective solubility products and the dissociation constant of the acid phosphate ions is not affected although for this particular case it would also depend on the relative amounts of calcium and phosphate in solution. The case is, however, a hypothetical one for, as previously stated, the solid phase is neither CaHPO_4 nor $\text{Ca}_3(\text{PO}_4)_2$ but a complex basic phosphate of whose ionisation we have no certain knowledge.

1. Lowering of the concentration of calcium ions in the plasma either as the result of a diminution in the total calcium content or of a disturbance in the equilibrium between the ionised and un-ionised portions thereof.

2. Lowering of the concentration of the phosphate ion corresponding with the bone phosphate (probably hydroxyapatite), either as a result of a diminution in the total inorganic phosphate or of a shift in the equilibrium between the various phosphate ions (change of p_H).

3. Failure of the cartilage and bone cells to secrete the enzyme.

4. Deficiency in the supply of the phosphoric ester which is normally present in the blood and is hydrolysed by the bone enzyme.

5. Alteration in the permeability of the corpuscular membrane, capillary wall or cartilage.

6. Failure of the mechanism (if such exists) by which the p_H of the tissue fluid is raised within the cartilage.

The occurrence of either (1) or (2) unaccompanied by an equivalent rise in the concentration of the other ion would result in the product of the ionic concentrations falling below the solubility product (k) of the bone phosphate and the plasma becoming unsaturated with respect to this substance. Deposition of the phosphate might still occur if the local increase in the inorganic phosphate resulting from the enzymic hydrolysis of the phosphoric ester were sufficient to raise the product of the ionic concentrations above k , but the amount of the calcium salt deposited would be reduced.

Failure either of the enzyme or of the specific phosphoric ester would result equally in deficient calcification since the necessary local increase in the phosphate concentration would not occur.

The phosphoric ester is present chiefly in the corpuscles and the conditions under which it passes into the plasma and cartilage fluids are not fully understood, but it is clear that the supply of this ester to the site of ossification might fail owing to changes in the permeability of the membranes (of the corpuscles etc.) even though the total amount present in the whole blood was not reduced.

It is conceivable also that changes in permeability of the cartilage or of the capillary walls might, by affecting the Donnan equilibrium, alter the relative concentrations of calcium and phosphate ions in the plasma and cartilage fluid.

The participation of the bone enzyme in the ossification process has been supported by a considerable weight of evidence but the further suggestion of a possible mechanism by which the p_H is locally raised is a teleological speculation and arises mainly from the evidence of the high optimum p_H of the enzyme. No direct proof of such a mechanism has yet been found. Its effect would be to increase the activity of the enzyme and, independently of this, to favour the deposition of calcium phosphate and of calcium carbonate.

It is obvious that a deficiency either of calcium or of phosphate in the diet, resulting in a lowered concentration of these substances in the blood, will

adversely affect calcification of the skeleton and may be a contributory cause of this defect in rickets, but the evidence at present appears to show that in well marked cases of rickets the concentration both of total calcium and of inorganic phosphate in the blood may be normal. A considerable proportion of the calcium is certainly present in un-ionised forms and will play only an indirect part in determining the precipitation of calcium salts. The investigation of the relation between the ionised and un-ionised calcium in plasma is a difficult but very attractive problem which we have not touched in the present paper but are hoping to attack later.

The inorganic phosphate is probably very largely ionised but the concentration of the particular anion corresponding with the solid phase (bone phosphate) must in any case be very small. It will be affected chiefly by the p_H of the fluid but to some extent also by the various cations present.

The bones of rachitic rats have been shown to contain at least as much enzyme as normal bones [Robison, 1923] and have been used to demonstrate the deposition of calcium phosphate *in vitro*, by immersing them in solutions of phosphoric esters. Failure to calcify is certainly not due to lack of enzyme. A deficiency in the amount of the specific phosphoric ester was however an attractive possibility and its investigation formed the chief part of the work here described.

EXPERIMENTAL.

Effect of Diet.

The first series of experiments was planned and carried out early in 1923 in collaboration with Dr Goldblatt before it was known that the organic acid-soluble phosphorus compounds in blood consist of at least two esters, only one of which, the smaller fraction, is hydrolysed by the bone enzyme [Kay and Robison, 1924].

A considerable number of rats was divided into groups and fed on various diets deficient in the fat-soluble factor, calcium or phosphorus or in two of these constituents, normal controls being provided for each group. When growth had ceased or rickets developed the animals were bled and killed under ether anaesthesia and estimations of the inorganic phosphate and total acid soluble phosphorus in the whole blood were carried out by the Briggs modification of the Bell-Doisy method. The fact that the inorganic phosphate is increased *in vivo* during ether anaesthesia and *in vitro* when the blood is laked with water was not discovered until after these experiments were completed [Martland and Robison, 1924] and it is very probable that the values obtained were all about 10 % too high. The averages for each group should however be comparable. The values for the total P should be correct. The results are shown in Table I. Except in the case of those rats which were fed on the McCollum diet there is no evidence that the deficiency of the fat-soluble factor results in a lowered concentration of inorganic phosphate in the whole blood

or that the addition of cod-liver oil to such a diet causes any appreciable increase. The average organic acid-soluble phosphorus is, however, regularly increased by the addition of cod-liver oil to the diet, although the amount of the increase is small and within the limits of variation found in individual rats on the same diet.

The rats fed on the McCollum diet No. 3143 showed an abnormal behaviour in this respect. The inorganic phosphate level was markedly below that found with any other of these deficient diets but was raised by nearly 70 % when cod-liver oil was given, although the total P did not change. The values obtained are not unlike those found by other investigators using this diet.

The low availability of the phosphorus derived from the cereals in this diet is well shown by comparison of the results obtained with those of Group IV in which the animals received a much smaller amount of phosphorus but chiefly in the form of inorganic phosphate, derived from the marmite. The concentration of inorganic phosphate in the blood was here nearly normal and was not increased by the addition of cod-liver oil to the diet.

Diets.

F. Heated casein, starch, cotton seed oil, marmite, lemon juice, salt mixture, mixed to a stiff paste with distilled water.

G. Same as F, with omission of calcium from the salt mixture.

E.W. Cooked egg-white replaced casein in diet F and a salt mixture free from phosphate was used.

McCollum 3143. Wheat, maize, wheat gluten, gelatin, calcium carbonate, sodium chloride, mixed to a stiff cake with distilled water.

Table I.

Group	No. of rats in group	Diet	% of Ca and P in diet as given to animals		Acid-soluble P compounds in 100 g. whole blood		Histology
			Ca	P	Inorganic mg. P	Organic mg. P	
I a	11	F (-A)	0.25	0.54	8.2	26.7	Mild rickets or osteoporosis
I b	11	F + C.L.O.	0.25	0.54	8.2	29.3	Normal with slight osteoporosis in some cases
II a	5	G (-A - Ca)	0.04	0.59	8.4	26.0	One normal, others with osteoporosis or osteomalacia
II b	3	G, + C.L.O. (-Ca)	0.04	0.59	8.5	28.4	Normal or osteoporosis
III a	5	E.W. (-A, -P)	1.08	0.08	4.5	20.5	Mild rickets or osteoporosis
III b	3	E.W. + C.L.O. (-P)	1.08	0.08	4.3	24.7	Nearly normal
III c	3	E.W. + C.L.O. + phosphate	1.08	0.54	5.8	27.3	Nearly normal
IV a	4	McCollum 3143	0.76	0.19	3.1	19.0	Rickets mild and florid
IV b	3	McCollum 3143 + C.L.O.	0.76	0.19	5.2	17.0	Nearly normal, some osteoporosis
IV c	3	McCollum 3143 + phosphate + C.L.O.	0.76	0.42	7.5	29.7	Nearly normal

Soon after these experiments were completed it was found that only a fraction (about 20 %) of the phosphoric esters is hydrolysable by the bone enzyme and it became apparent that increases (2-3 mg. P) such as were observed when cod-liver oil was added to the diets might, if occurring entirely in this fraction, represent very significant changes. A further series of experiments was therefore planned in which the amount of phosphoric ester hydrolysable by the bone enzyme should be estimated according to the method

Table II.

No. of exp.	No. of rats	Diet	Acid-soluble P compounds in 100 g. blood			Analysis of bones			
			Inorganic mg. P	Organic mg. P	Hydrolysed by bone enzyme mg. P	H ₂ O %	Ca % of dry bone	P % of dry bone	Ca : P
1	6	McCollum 3143	2.5	20.1	3.6				
	5	Do + C.L.O.	4.1	20.2	4.2				
9	3	McCollum 3143	2.6	24.9	5.1	62.5	12.11	5.40	2.24
	3	Do + C.L.O.	4.6	21.1	3.1	56.6	16.17	7.44	2.17
16	2	McCollum 3143	3.2	19.3	3.2	—	14.5		
	2	Do + 10 cc. milk	5.1	18.8	3.2	—	16.2		
18	2	McCollum 3143	2.1	24.3	4.5	54.8	16.30	7.22	2.26
	2	Do + oxidised C.L.O.	5.4	27.7	3.9	47.8	18.37	8.46	2.17
3	3	F	5.1	27.0	3.4	47.2	16.46	7.72	
	2	F + irradiation	5.1	27.6	4.4	44.6	20.36	9.27	
	2	F + C.L.O.	5.6	27.3	4.6	40.8	21.95	10.64	
5	4	F	5.1	26.6	5.6	47.5	18.69	8.68	
	2	F + irradiation	5.3	25.4	5.3	44.9	21.06	9.75	
	3	F + C.L.O.	5.3	28.0	6.4	39.3	21.60	9.92	
12	6	F	6.5	30.3	10.0	45.4	16.38	7.59	2.16
	6	F + C.L.O.	4.9	32.5	8.2	39.2	21.13	9.71	2.18
13	4	F	7.0	27.3	7.6	46.5	15.95	7.42	2.15
	2	F + C.L.O.	5.4	34.0	7.8	39.6	20.48	9.45	2.17
14	4	F ₁	4.6	29.5	5.9	41.5	19.73	9.18	2.15
	4	F ₁ + C.L.O.	4.9	34.3	6.0	40.5	21.08	9.87	2.14
	4	F ₁ + sodium phosphate injections	4.3	32.1	6.5	45.6	19.54	9.15	2.14
	4	F ₁ sodium glycerophosphate injections	4.7	29.5	5.2	42.4	20.21	9.36	2.16
15	2	F	4.5	28.2	5.8	46.7	19.06	9.00	2.12
	2	F + butter fat	4.6	30.5	5.3	41.4	20.86		
	2	F + oxidised C.L.O.	4.4	31.3	5.7	39.1	21.32	9.84	2.17
17	2	F	3.8	24.0	5.4	54.4	16.91		
	2	F + unsaponifiable matter of C.L.O.	4.5	28.0	4.6	46.6	19.80		
8	2	E.W. + casein	5.9	30.1	6.3	39.3	17.12		
	2	Do + C.L.O.	5.7	35.0	7.1	39.7	18.61		
	2	Do + 5 g. spinach	5.8	26.9	7.2	41.0	19.86		
	2	Do + 5 g. cabbage	5.9	37.8	6.6	40.0	19.05		
11	4	F	8.6	27.6	4.9	—	15.3		
	3	F + 0.1 g. spinach	8.8	31.1	4.4	—	15.9		

Diet F₁ (Exp. 14) contained 0.54 % of calcium and 0.54 % of phosphorus but was otherwise the same as diet F.

Diet E.W. + casein (Exp. 8) contained 0.67 % of calcium and 0.73 % of phosphorus.

The calcium and phosphorus contents of the other diets are given in Table I.

In Exp. 16 the 10 cc. milk increased the calcium content of the diet by about 10 % and the phosphorus content by about 30 %.

described by Kay and Robison [1924]. Rats from the same litter and in most cases from the Lister stock were used and the males and females were divided as equally as possible between the groups. Three experiments were also made on guinea-pigs but were not very satisfactory and the results have been omitted from the Table. The animals were killed in fasting condition¹ by severing the carotid and jugular without anaesthetic and the blood was taken directly into 1 % trichloroacetic acid to stop the action of the phosphoric esterase in the blood. This precaution, however, introduced a new difficulty. The cloudiness which occasionally appears on addition of the ammonium molybdate solution in estimating phosphate by the Briggs method, became more frequent and intense. The cause was finally traced to the presence of protein cleavage products formed by the action of a pepsin-like enzyme in the blood. The hydrolysis occurs much more rapidly in 1 % trichloroacetic acid than when the blood is laked with water. We continued to use the 1 % trichloroacetic acid for laking but added the further quantity of the acid as soon as possible to precipitate the proteins. This difficulty was only encountered in the case of rat's blood. Human blood and that of rabbits appear to contain much less "pepsin." The results of these experiments are set out in Table II.

Inorganic phosphate. As in the previous series the concentration of inorganic phosphate in the blood of the rats on diet 3143 was abnormally low but was raised almost to normal by the addition of cod-liver oil. The figures for the groups receiving other diets containing a sufficiency of inorganic phosphate provide no evidence that the deficient calcification of the bones produced in absence of the fat-soluble factor is associated with an abnormally low concentration of inorganic phosphate in the blood or that this concentration is raised by cod-liver oil or other sources of the vitamin. The figures for the normal controls show considerable variations and are obviously affected by other factors of which we have insufficient knowledge.

Organic phosphoric esters. In the groups on diet 3143 the total amount of phosphoric esters was again not raised by cod-liver oil except in one experiment (18) in which the rats were exceptionally well grown, being older than usual when placed on the diet. On the other diets the total amount of phosphoric esters was increased by the addition of cod-liver oil, by oxidised cod-liver oil, by the unsaponifiable fraction of the oil and by cabbage. Against the conclusion that this increase is associated with the improved calcification of the bones must be placed the fact that in Exp. 3 the amount of the increase was vanishingly small although the improvement in the calcium content of the bones was very marked. On the other hand, in Exp. 11 a decided increase in the amount of the esters was accompanied by very slight improvement in the bones. Irradiation appeared to have little effect on this value.

As to the phosphoric ester which is hydrolysable by the bone enzyme, we can find from these figures no satisfactory evidence of any abnormally low concentration in the blood of rats on —A diets nor of any significant

¹ Except in Exp. 14, in which the rats were killed two hours after feeding and injections.

regular increase connected with the addition of cod-liver oil to the diet. Whether the increase in the amount of the other ester which is not hydrolysable by the bone enzyme may have any indirect effect on the calcification of the skeleton we are not at present prepared to offer an opinion. Possibly the investigation at present in progress of the phosphoric-esterase of the blood may throw some light on this question. The activity of this enzyme appears to be subject to very delicate control by the p_H of the blood and to influence in a very marked degree the inorganic phosphate level [Martland, 1925].

Effect of injecting sodium glycerophosphate.

As an additional method of discovering whether the defective calcification is due to a lack of the specific phosphoric ester it was further proposed to study the effect of injecting sodium glycerophosphate into rats on a diet deficient in the fat-soluble factor. Unfortunately, owing to the fact that the experiment (Exp. 14) was carried out during the summer months with rats from a particularly strongly growing stock, the controls failed to exhibit very marked symptoms and the percentage calcification of the bones was not greatly below that of the normal animals. Two litters of rats, divided into four groups each of four animals, were placed on the experimental diets when 21 days old, and 10 days later the injections of sodium glycerophosphate (1 cc. $M/5$ solution daily, subcutaneously) were commenced in one group. A second group received injections of equivalent amounts of sodium phosphate (Na_2HPO_4) while a third received no injections. The fourth group received cod-liver oil (by mouth) from the beginning to the end of the experiment which lasted 57 days. Analyses were made in duplicate of the bones of the hind legs of all animals of each group collectively and the blood was also investigated (Table II). The average Ca content of the dry bones of the groups receiving sodium glycerophosphate injections was 2.4 % higher than that of the groups receiving none and 3.4 % higher than that of the group receiving sodium phosphate injections which was even lower than that of the controls. Cod-liver oil increased the calcium content of the bones by 6.8 % above that of the —A group. The evidence of this experiment, that the injection of sodium glycerophosphate produces some increase of calcification while that of sodium phosphate has no such effect, could not, in view of the small increases observed, be considered satisfactory without further confirmation. We did not, however, continue these experiments as the subject was being very thoroughly investigated by Korenchevsky and Carr [1925]. As the result of their experiments they conclude that sodium glycerophosphate has a definite effect in increasing the calcium content of the bones of such rachitic rats, while injections of sodium phosphate produce less or no improvement. They find however that calcium glycerophosphate produces a much greater improvement than the sodium salt.

If we may draw any conclusion from these results and from our own, it is that while a deficiency of the specific phosphoric ester is not the cause of

the defective calcification, an excess of this ester may go some way towards counteracting the lack of the unknown responsible factor. The marked effect of calcium glycerophosphate suggests but does not prove that the deficiency is in the calcium ions.

That the sodium salt of the phosphoric ester possesses some beneficial effect not shown by sodium phosphate may be taken as additional evidence of the participation of the bone enzyme in the process of calcification.

The Ratio of Ca and P in the bones.

Both the phosphorus and calcium were determined in the bones of the experimental animals as it was thought possible that variations in the ratio Ca : P might throw light on the deficient factor. This ratio would be 1.93 for $\text{Ca}_3(\text{PO}_4)_2$, 2.15 for calcium carbonato-phosphate ($\text{Ca}_3\text{P}_2\text{O}_8$) $\cdot\text{CaCO}_3$ or for hydroxyapatite ($\text{Ca}_3\text{P}_2\text{O}_8$) $\cdot\text{Ca}(\text{OH})_2$ (Bassett), and 2.37 for a mixture of 1 mol. hydroxyapatite with 1 mol. CaCO_3 (9 %). If the carbonate and phosphate are precipitated independently a deficiency of the phosphate ion might give rise to bones showing a higher ratio of Ca : P if the factors governing the precipitation of CaCO_3 were unaffected. A deficiency of the Ca ion would not necessarily alter the ratio, unless the calcium were replaced by magnesium. Calculations based on the published analyses did not furnish very hopeful results. Thus Gabriel's [1894] analyses give ratios of 2.27 for human bones, 2.24 for ox bone and 2.19 for goose bone; Gassmann's [1910] analyses of bones of normal and rachitic children give 2.24 and 2.16 respectively, the lower ratio in the rachitic bone being accounted for by an increase in Mg from 0.1 % to 0.64 % of the dry bone. Our own analyses were carried out in duplicate on aliquot portions of solutions obtained by igniting the bones of hind legs with nitric and sulphuric acid. The Ca was estimated by the method of Aron and Sebauer and P by that of Neumann. It would have been better had the bones first been extracted with alcohol and ether but it was found by actual experiment that the amount of P thus extracted does not represent more than 0.2 %–0.3 % of the total P of the bone so that the error introduced in the ratio Ca : P is very small. In Exps. 3 and 5 the Ca and P estimations were done on separate bones and the ratios are therefore of no significance.

In the experiments in which the basal diets, F and F_1 , were not deficient in phosphate the ratios show a considerable degree of constancy, all lying close around 2.15 (± 1 %). There is a general tendency for the ratio to be slightly lower in groups on $-A$ diet than in those receiving cod-liver oil. The ratios for the groups receiving McCollum's low phosphorus diet 3143 are decidedly higher (2.25) but are reduced to the normal figure by cod-liver oil. Without wishing to press these figures too far it may be said that they would accord with the supposition that in rats fed on diet 3143 the deficiency is chiefly one of phosphate ions while on diets deficient only in the fat-soluble factor it is the concentration of Ca ions which falls below the normal.

In such a complex problem as that of rickets we do not wish to suggest that our conclusions have necessarily any wider application than for the particular cases investigated, *i.e.* defective calcification produced in rats by

diets deficient in the fat-soluble factor. It is hoped to extend the investigations to the blood of other animals and of children in rachitic conditions and some data have already been obtained in this direction, but the need of a closer study of the various forms of calcium in the blood seems to be even more clearly indicated.

We are greatly indebted to Dr M. Boas, Dr H. Chick and to Professor Drummond for allowing us to investigate the blood of the animals in certain of their experiments (Exps. 8, 11, 16, 17) and for permission to quote their analyses of the bones of these animals.

SUMMARY.

The blood of rats fed on diets containing a sufficiency of calcium and inorganic phosphate but deficient in the fat-soluble factor contains a normal amount of inorganic phosphate and of the phosphoric ester hydrolysable by the bone enzyme, and these are not increased by the addition of cod-liver oil to the diet.

The amount of the phosphoric ester resistant to the action of the bone enzyme is usually increased by cod-liver oil or other sources of the fat-soluble factor but there appears to be no direct connection between this increase and the improvement in the calcification of the bones.

The defective calcification of the skeleton resulting from such diets is probably not due to any deficiency of inorganic phosphate or of the specific phosphoric ester in the blood, or of the enzyme in the bone by which the ester is hydrolysed.

An excess of hydrolysable esters (caused by the injection of sodium glycerophosphate) may however partly counteract the lack of the unknown responsible factor and producesomeimprovement in the calcification of the bones.

In rats fed on a diet (McCollum 3143) very deficient in inorganic phosphate the concentrations of inorganic phosphate and of phosphoric esters in the blood are below the normal. The inorganic phosphate level is raised almost to the normal by cod-liver oil but the amounts of the esters are not significantly increased.

The ratios of Ca : P in the bones support the view that in rats fed on this diet the defective calcification is due mainly to a deficiency of inorganic phosphate ions while this is not the case when the defect is caused by a lack of the fat-soluble factor alone.

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