CVI. POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION.

PART VI. PHOSPHORIC ESTERS IN BLOOD-PLASMA.

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The explanation of the process of calcification suggested in the first of these papers requires that a suitable phosphoric ester shall be supplied to those regions of bone and cartilage where ossification is taking place. It has been shown that one of the acid-soluble phosphoric esters occurring in blood fulfils the conditions of suitability in that it yields a soluble calcium salt and is hydrolysed by aqueous extracts of the bone enzyme, but the fact that these esters are almost entirely confined to the corpuscles[1] makes it necessary to enquire further into the possibility of this compound passing into the fluids bathing the cartilage cells. This possibility would not be excluded even if the ester were normally absent from blood-plasma, for a varying permeability of cell membranes must be assumed to explain many of the facts of physiology.

The amounts of organic acid-soluble P found by previous workers in normal plasma or serum are set out in Table I.

Table I.

Author	Animal	Organic P in serum (mg. per 100 cc.)		
		Lowest	Highest	Average
Greenwald* [1916]	Sheep	0·43	1·06	0·68
,,	Pig	—	—	0·46
Feigl† [1917]	Human	0·20	0·90	0·51
Bloor [1918]	Men	0·13	1·30	0·54
,,	Women	0	1·30	0·40
Jones and Nye‡ [1921]	Children	(–2·0)	2·0	0·44
McKellips, De Young and Bloor [1921]	Human adult	0·06	1·3	0·48
,, ,,	Human infant	0·40	3·6	2·21
Buell [1923]	Dog	—	—	Negligible
Plass and Tompkins [1923]	Human maternal	0	1·3	0·73
,, ,,	Human foetal	0·30	3·1	1·23
Stanford and Wheatley§ [1925, 2]	Human	—	—	None

* Greenwald found that this organic P was not readily dialysable.

† Feigl has also investigated the organic P content of human serum in many pathological conditions. [See Feigl, 1920.]

‡ In view of the large experimental error shown by these results no great importance can be attached to this average value.

§ In four out of five plasmas examined, Stanford and Wheatley [1925, 2] actually found "unknown P" amounting to 0·5–0·8 mg. per 100 cc., but this does not appear to have been acid-soluble. The probable error of their method, which they give as ±5 % [1925, 1] would largely mask the small amounts of P which might be present.

[1] Ether-soluble phosphoric esters (phospholipins) occur in considerable amount in both plasma and corpuscles but these are not hydrolysed by the bone phosphatase.

The accuracy with which the total P can be estimated is the decisive factor in this question, since the organic fraction can only be determined by difference (total P − inorganic P), and it was therefore necessary to reduce the experimental error as far as possible and to have precise knowledge of its limits before we could place any reliance on our own results.

METHOD.

In a previous paper [Martland and Robison, 1924] certain possible sources of error in the estimation of P by the Briggs method were pointed out and it may be of use to describe the exact procedure now employed after 2 years' further experience. It should be added that the slight modifications introduced involve no radical change in this very useful method. The following solutions are used:

A. Concentrated H_2SO_4 30 cc.
   Water to 100 cc.

B. Ammonium molybdate 10 g.
   Water to 100 cc.

C. Sodium sulphite 20 g.
   Quinol 0·5 g.
   Water to 100 cc.

The sulphuric acid and ammonium molybdate are now added separately in estimating both inorganic P and total P, in order that the same solutions may be used throughout. If the molybdate solution is kept for any length of time a precipitate may form and if the amount of this is large the solution should be rejected. For this reason the original Briggs solution is preferable for routine use when only inorganic P is to be estimated.

From plasma or serum the proteins are precipitated in a dilution of 1 in 5 with 3·5 % trichloroacetic acid. Whole blood or corpuscles are first laked with 1 % trichloroacetic acid and the proteins are then precipitated by raising the concentration of the acid to 3·5 %. Filtration is carried out after 15 minutes through a P-free filter paper (No. 30 Whatman), slight suction being permissible.

Inorganic P. 5 cc. of the filtrate (equivalent to 1 cc. plasma or serum) are pipetted into a 15 cc. graduated flask, a suitable volume of the standard KH_2PO_4 solution (1 cc. = 0·01 mg. P) being measured into a similar flask. Both the solutions are diluted to about 10 cc. with water and 1 cc. each of solutions A, B, C are then added, the flasks being gently shaken between each addition. After dilution to the mark the contents are thoroughly mixed and the colorimeter readings are taken after half an hour. It is advisable to calibrate the graduated flasks and to make a correction for the volume error, which is frequently considerable.

Total acid-soluble P. We have abandoned the use of the nitric-sulphuric acid mixture for ignition owing to the risk of low results due either to incomplete oxidation or, if a higher temperature be employed, to combination of

phosphoric acid with the glass. The latter can be easily demonstrated if a flask which has been etched in this manner is rinsed with water and then warmed with a solution of ammonium molybdate, ammonium nitrate and sulphuric acid as in the Neumann method for P estimation. The presence of phosphate is shown by the yellow precipitate formed on the etched surface. Such loss chiefly occurs through the overheating of those parts of the glass which are just above the liquid, but the danger is not entirely obviated by the use of narrow boiling tubes and small flames. Pyrex tubes heated in a metal bath below the boiling point of sulphuric acid have sometimes shown such etching.

The danger of incomplete oxidation at lower temperatures can be overcome by repeated ignitions with small additional amounts of nitric acid[1], but we have found Baumann's [1922] method of ignition with 30 % hydrogen peroxide and sulphuric acid both simpler and more satisfactory. With this agent oxidation is rapid and complete at such moderate temperatures as involve no risk of loss by etching. Baumann found it necessary to re-distil the hydrogen peroxide (Merck's "superoxol") which he employed. We have used Merck's "perhydrol" and up to the present have found it free from phosphorus. Other makers' preparations of similar strength have contained phosphorus in large amounts.

The procedure is as follows. 5 cc. of the trichloroacetic acid filtrate are measured into a Pyrex boiling tube; 1 cc. of solution A is added and the liquid is concentrated on the convenient electric heater described by Stanford and Wheatley [1925, 1] until slight charring takes place. 0·1 cc. of Merck's "perhydrol" is then added and the ignition continued for about 4 minutes, until the dense white fumes first evolved have cleared. The acid residue should be quite colourless, but if any charring persists a further drop of perhydrol should be added and the ignition repeated. When the tube has cooled the contents are rinsed with successive small quantities of water into a 15 cc. flask and 1 cc. each of solutions B and C are added[2], the standard being made up as before with all three solutions.

Factors affecting the accuracy of the method.

Proportionality of colour produced to phosphate present. We cannot agree with Stanford and Wheatley's [1925, 1] statements on the lack of proportionality between colour produced and phosphate present. We have no experience of Stanford's [1923] dilution colorimeter, but with an instrument of the Dubosc type (Bausch and Lomb) we find, as stated in our previous paper, that the intensity of the colour is proportional to the amount of phosphate within very wide limits. The following results may be quoted in support of this conclusion.

[1] The addition of sugar solution after ignition with nitric acid is not necessary. Such traces of nitric acid as remain do not affect the colour intensity.

[2] If the final ignition has been unduly shortened traces of peroxide (persulphuric acid) may remain and form yellow permolybdic acid on addition of solution B. This is reduced by the sulphite but may cause the results to be low.

Amounts of inorganic phosphate varying from 0·08 to 0·02 mg. P were compared with a standard containing 0·1 mg. P, the maximum difference corresponding with a ratio of 1 : 5.

The amounts of P calculated from the colorimeter readings are shown below:

mg. P present	·0800	·0600	·0400	·0200
mg. P found	·0805	·0603	·0400	·0196

It is obvious, however, that with the usual colorimeter scale the percentage accuracy of the readings will be greatest when both unknown and standard are of nearly the same intensity, and this will naturally be chosen as the optimum condition.

Effect of the degree of acidity on the colour intensity. We confirm our previous statement that although the rate of development of the colour depends on the degree of acidity, small variations (± 15 %) in the amount of acid, under the conditions given, have no significant effect on the colour produced in ½ hour to 2 hours. In particular, the presence of trichloroacetic acid in amounts up to 0·25 g. (*i.e.* a final concentration of 0·1 *N*) does not cause any inaccuracy in the result as is shown by the following estimations of inorganic phosphate.

Trichloroacetic acid added (cc. 25 % sol.)	0	0·5	1·0
mg. P present	0·0500	0·0500	0·0500
mg. P found	standard	0·0500 0·0506	0·0500 0·0499

The use of several standard solutions containing varying amounts of trichloroacetic acid, as advocated by Stanford and Wheatley, is thus an unnecessary complication.

Error of the method.

In routine estimations of inorganic phosphate where the amount present is about 0·05 mg. P we find that the error does not usually exceed ± 0·0005 mg. (± 1 %). By meticulous attention to every detail this error may be reduced by half. As in all colorimetric work, the possibility of occasional errors of relatively large dimensions must be reckoned with and these can only be guarded against by rigid controls.

The error of the method for total P was investigated by carrying out series of estimations on standard solutions both of KH_2PO_4 and of sodium glycerophosphate. In this way errors due to losses of P or other effects of the ignition were distinguished from such as might be due to incomplete oxidation of the ester. A large number of duplicate estimations of total P in blood filtrates has also been carried out. The common practice of determining the accuracy of a method by the recovery of added phosphate from blood or serum is unsatisfactory because it does not exclude the possibility of constant errors which may even be of large dimensions. We have frequently obtained duplicate results showing excellent agreement although wide of the correct value.

The standard glycerophosphate solution was prepared as follows. The

P contents of an approximately $M/30$ solution of sodium glycerophosphate and of a standard KH_2PO_4 solution (1 cc. = 1 mg. P) were estimated by Neumann's method, the mean of at least four concordant results being taken. Each solution was then diluted 1 in 100, using a standard pipette and flask. The exact value of the diluted glycerophosphate solution in terms of the diluted KH_2PO_4 solution was then known and the latter was used as standard in the colorimetric estimation.

We give a summary of the latest series of tests.

KH_2PO_4. Estimations carried out as for total P (perhydrol ignition).

Amount of P present. 0·1000 mg. No. of estimations 23. Average of all results 0·0995 mg. Extreme limits 0·1003 and 0·0986, but 19 results lay between 0·1000 and 0·0990 (± 0·5 % of average).

Sodium glycerophosphate.

(i) Amount of P present 0·1170 mg. No. of estimations 22. Average results 0·1165 mg. Extreme limits 0·1174 and 0·1154, but 17 results lay between 0·1171 and 0·1159 (± 0·5 % of average).

(ii) Amount of P present 0·0351 mg. No. of estimations 5. Average results 0·0348 mg. Extreme limits 0·0351 and 0·0347, but 4 results lay between 0·0348 and 0·0347 (± 0·3 % of average).

From these and many other tests we conclude that the results obtained by this method will probably be below the correct value by not more than 1 % and that at most the error should not exceed + 0·5 % to − 1·5 % of this value. Duplicate results on blood filtrates also fall within these limits.

Phosphorus in serum.

Having satisfied ourselves that we could determine the inorganic P and total acid-soluble P in 1 cc. of serum with sufficient accuracy to detect differences greater than 1 % we set out to examine blood taken from different animals under varying conditions so as to discover whether organic acid-soluble P actually occurs in the serum, and if so whether and under what conditions the amount varies.

No anti-coagulant was used as we wished to avoid any possible effect of such additions on the partition of the P compounds between corpuscles and plasma. The blood was centrifuged at once so that the corpuscles were thrown down before clotting began. The serum was separated by gentle pressure on the fibrin clot and again centrifuged. Sometimes it was possible to pipette off the plasma before the clot formed, but in any case it was centrifuged a second time to remove any residual corpuscles. The presence of 0·1 % of the red corpuscles—intact or laked—would account for about 0·04 mg. organic P per 100 cc. in human serum and 0·05–0·07 mg. in the case of rats or rabbits. Microscopical examination of the serum showed that the number of corpuscles remaining was always far below this figure, while the absence of haemolysis was shown by the colour and by spectroscopic examination. Haemolysis

corresponding with less than 0·05 % laked corpuscles would not, however, be easy to detect. We are satisfied that if any of the organic P is to be ascribed to such adventitious causes the amount cannot exceed 0·04 mg. per 100 cc. serum.

The possibility that the organic P was not of the acid-soluble type, but was due to traces of phospholipins which had escaped precipitation with the proteins, was also examined. 50 cc. of trichloroacetic acid filtrate (equivalent to 10 cc. serum) which contained 0·044 mg. organic P (0·44 mg. per 100 cc. serum) was extracted with ether. After distilling off the ether the residue was examined but was found to contain no P, thus confirming our previous observations on this point.

Tables II and III show the amounts of inorganic and organic P found in the serum of blood taken from normal subjects in the fasting and absorptive states, after the administration of large amounts of phosphates and after injection of certain hormones.

Table II. *Acid-soluble P in human serum.*

(Blood taken from arm vein in all cases)

Subject	Conditions of test	Acid-soluble P in serum (mg. P per 100 cc.) Inorganic	Organic	Organic as % of total
Man [R. R.]	After 17 hours' fast	3·42	0·34	9·0
	1½ hours after food	3·37	0·17	4·8
,,	After 21 hours' fast	3·81	0·24	5·9
	2 hours after food	3·74	0·25	6·3
,,	2 hours after food	2·50	0·25	9·1
	0·5 cc. pituitrin injected after the previous sample was taken, 2nd sample 50 minutes later	2·72	0·35	11·1
,,	2½ hours after food	3·58	0·18	4·8
	10 g. NaH_2PO_4(+ $NaHCO_3$) then taken in 1 % solution, 2nd sample 1 hour later	5·83	0·20	3·3
,,	30 g. NaH_2PO_4(+ $NaHCO_3$) taken in three doses at hourly intervals, blood withdrawn 1 hour after last dose	7·14	0·66	8·5
,,	25 g. NaH_2PO_4(+ $NaHCO_3$) taken in two doses before and after meal, blood withdrawn ¾ hour after last dose	5·49	0·35	6·0
,,	10 g. NaH_2PO_4(+ $NaHCO_3$) taken before meal, blood withdrawn 3½ hours later	3·91	0·04	1·0
Woman	After 13 hours' fast	3·82	1·27	25·0
,,	1½ hours after food	3·72	0·02	0·5
,, syphilitic	3 hours after food	3·80	0·14	3·6
,, ,,	2½ hours after food	3·76	0·16	4·1
,, ,,	1½ hours after food	4·26	0·13	3·0
,, diabetic	After 13 hours' fast	3·68	0·08	2·1
,, ,,	After 13 hours' fast	3·81	0·05	1·3

It must therefore be recognised that organic acid-soluble compounds of phosphorus are normal constituents of blood-serum and therefore of blood-plasma. The values here shown are probably too low on two accounts.

(1) The error in the estimation of total P already referred to. The average correction under this heading might amount to 1 % of the total P, although it would not be justifiable to apply this correction to any individual result.

(2) The hydrolysis of the phosphoric ester during the interval (40–50 minutes) between the withdrawal of the blood and the precipitation of the serum proteins. Experiments described below show that traces of phosphatase are present in serum and that as much as 0·1 mg. organic P might be hydrolysed in this time.

Table III. *Acid-soluble P in rabbit serum.*

Age of animal	Type of blood and conditions of test	Acid-soluble P in serum (mg. P per 100 cc.) Inorganic	Organic	Organic as % of total
3 weeks	Venous (ear vein)	11·20	0·28	2·4
4 ,,	Mixed	7·96	0·13	1·6
6 ,,	Arterial (cardiac puncture)	7·00	0·20	2·8
Adult	Mixed	4·32	0·30	6·5
,,	,,	8·52	0·44	4·9
,,	,,	3·18	0·31	8·8
,,	Arterial (cardiac puncture)	5·60	0·26	4·4
,,	Venous (ear vein)	4·53	0·42	8·5
,,	Venous (ear vein) 1st sample taken after feeding	7·94	0·36	4·3
,,	Animal then allowed to drink a solution of Na_2HPO_4 (0·35 g. P) + 20 g. glucose. 2nd sample taken 2½ hours later	7·91	0·48	5·7
,,	Repetition of above exp. 1st sample before taking Na_2HPO_4, etc.	7·97	0·29	3·6
	2nd sample after taking Na_2HPO_4, etc.	7·86	0·43	5·5
,,	Venous (ear vein). 1st sample taken after feeding	4·73	0·12	2·5
	Animal then given intramuscular injection of 0·5 cc. pituitrin (P.D.). 2nd sample taken 50 minutes later	5·68	0·22	3·7
,,	Repetition of above exp. but with two injections of 0·5 cc. pituitrin at an interval of 1 hour: 1st sample before injections	4·15	0·29	6·5
	2nd sample 15 minutes after 2nd injection	2·99	0·34	10·2

We conclude that the amounts of organic acid-soluble P present in the circulating plasma would probably be at least 0·1 mg. more than the values shown in the tables.

It is clear that the amount is by no means constant. In one subject (R. R.) 0·04–0·66 mg. per 100 cc., representing from 1 % to 11 % of the total acid-soluble P was found at different times, while as much as 1·27 mg. or 25 % of the total P was found in another case. Serum obtained from this subject on a later day contained only 0·02 mg., or 0·5 % of the total P, in organic form. The results do not, however, throw much light on the cause of these variations and we do not feel justified in forming any opinion on this question until further work has been done.

Experiments carried out with whole blood *in vitro* have shown that during the hydrolysis or synthesis of phosphoric esters which can be brought about by slight variations in the p_H of the blood [Martland, 1925], the changes in the amount of inorganic and organic P occur primarily in the corpuscles, and that the concentration of inorganic phosphate in the plasma is only slightly altered. The corpuscular membrane seems to be not readily permeable to inorganic phosphate ions and considerable difference in concentration may

thus ensue. This is probably the explanation of the different conclusions which have been formed as to the relative amounts of inorganic phosphate in corpuscles and plasma. The phosphoric ester, or esters, concerned in these reactions belong mainly to the fraction which is not hydrolysed by the bone enzyme [Martland, Hansman and Robison, 1924]. After many experiments, no satisfactory evidence has been obtained of any increase in the amount of organic P in the plasma during these changes which are brought about by the activity of enzymes in the corpuscles, nor have we any evidence of synthesis occurring in serum by the action of enzymes there present.

Hydrolysis of the phosphoric esters by the plasma phosphatase.

Blood-plasma contains small amounts of a phosphatase similar to that found in bone. Like the latter it hydrolyses sodium glycerophosphate, hexosemonophosphate and hexosediphosphate and has an optimum p_H in the region of 9, whereas the optimum for the corpuscle phosphatase is about 6. At the optimum p_H and in presence of excess of glycerophosphate or hexosemonophosphate serum of normal adult rabbits has been found to hydrolyse about 30 mg. P in 18 hours at 38°. The serum of a young rachitic animal showed a much greater activity (94 mg. P in 18 hours at p_H 8·4)[1].

This serum phosphatase was found to hydrolyse the organic P compounds of the serum itself, and although its activity at room temperature and at the p_H of the blood would be only a small fraction of its optimum, yet we have obtained evidence of small but definite hydrolysis of organic P during the time between the withdrawal of the blood and the precipitation of the serum protein. This loss may amount to about 0·1 mg. P as is shown by the following figures (mg. P per 100 cc.).

		Time after withdrawal of blood			
		Room temperature 50 minutes	Room temperature 1 hour 50 minutes	Room temperature 50 minutes + 3 hours at 38°	Amount hydrolysed
Serum 1	Inorganic P	3·41	3·53	—	—
	Organic P	0·32	0·22	—	0·10
Serum 2	Inorganic P	8·52	—	8·84	—
	Organic P	0·44	—	0·09	0·35

Action of the bone enzyme on the phosphoric esters in serum.

Human blood contains an average of about 20 mg. P per 100 cc. in the form of acid-soluble esters, of which about 6 mg. or 30 % is hydrolysed by the bone enzyme. The figures for rabbit blood are about 50 % higher in each case. The esters in the plasma might belong to either of these types or might be a mixture as in the corpuscles. The action of the bone extracts on these esters had been tested from time to time and the evidence seemed to show that the major portion was hydrolysed, but until recently these experiments did not

[1] Demuth [1925] has investigated the activity and optimum p_H of the serum phosphatase in various pathological conditions, using hexosediphosphate as substrate.

seem to us convincing on account of the small amounts of P involved, in comparison with the possible errors of estimation.

The following experiment carried out with all the precautions referred to above has justified the previous conclusion and shows that the whole of the organic P in this serum was hydrolysed in 3½ hours at 38° by the bone phosphatase.

The trichloroacetic acid filtrate, obtained as described above from the serum of an adult rabbit, was neutralised with sodium hydroxide.

30 cc. were treated with 0·5 cc. of a 20 % extract of young rabbit bones, the p_H being adjusted to 8·6. 10 cc. were removed immediately for estimation of total and inorganic P, and the remainder was kept at 38°, 10 cc. being removed after 1½ and 3½ hours. The P in the bone extract was separately estimated and was equivalent to only 0·68 mg. P per 100 cc. serum. This has been subtracted from the results which are shown below.

	0 hours	1½ hours	3½ hours
Total P	8·97	9·01	8·97
Inorganic P	8·58	8·89	9·06
Organic P	0·39	0·12	—

The whole of the organic P in the serum amounting to 0·39 mg. per 100 cc. was thus hydrolysed in 3½ hours under these conditions.

The partition of the hydrolysable ester between corpuscles and plasma would appear in this case to be roughly in the ratio of 25 to 1.

Summary.

The presence of small amounts of acid-soluble phosphoric esters in blood-plasma has been confirmed.

These esters are hydrolysable by the bone phosphatase.

REFERENCES.

Baumann (1922). *Proc. Soc. Exp. Biol. Med.* **20**, 171.
Bloor (1918). *J. Biol. Chem.* **36**, 49.
Buell (1923). *J. Biol. Chem.* **56**, 97.
Demuth (1925). *Biochem. Z.* **159**, 415.
Feigl (1917). *Biochem. Z.* **83**, 81.
—— (1920). *Biochem. Z.* **112**, 27.
Greenwald (1916). *J. Biol. Chem.* **25**, 431.
Jones and Nye (1921). *J. Biol. Chem.* **47**, 322.
Martland (1925). *Biochem. J.* **19**, 117.
Martland, Hansman and Robison (1924). *Biochem. J.* **18**, 1152.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
McKellips, De Young and Bloor (1921). *J. Biol. Chem.* **47**, 53.
Plass and Tompkins (1923). *J. Biol. Chem.* **56**, 309.
Stanford (1923). *Biochem. J.* **17**, 839.
Stanford and Wheatley (1925, 1). *Biochem. J.* **19**, 697.
—— —— (1925, 2). *Biochem. J.* **19**, 706.