XCVII. NOTE ON THE ESTIMATION OF PHOSPHORUS IN BLOOD.

By MARJORIE MARTLAND and ROBERT ROBISON.
From the Biochemical Department of the Lister Institute.

(*Received May 1st, 1924.*)

During the course of work carried out in this laboratory during the past eighteen months and involving a very large number of micro-estimations of inorganic and total phosphorus in blood, certain points of interest and sources of error have been noted and are here briefly set out.

Estimation of inorganic and total phosphorus in blood by the Bell-Doisy and Briggs methods.

The original Bell-Doisy [1920] method has been largely superseded by the modification introduced by Briggs [1922] which was free from the main objections raised against the former method. These were the rapid fading of the colour and the diminished intensity of the colour produced in presence of oxalate or citrate. There is another objection, which, so far as we are aware, has not been mentioned previously. The intensity of the final colour varies considerably with changes, in the degree of acidity of the solution in the first stage of the process. This is not so important in the estimation of inorganic phosphate where the acidity of standard and unknown may be equalised, but appreciable errors may occur in the estimation of total phosphorus after acid ignition since the amount of residual acid can only be gauged approximately and may differ from the amount added to the standard. The following examples illustrate the point.

(*a*)	1·0 cc. standard phosphate soln. (0·05 mg. P)		Colorimeter reading	20·0 mm.
(*b*)	,, ,,	+1 cc. 20 % trichloroacetic acid.	,, ,,	20·95 ,,
(*c*)	,, ,,	+0·1 cc. conc. H_2SO_4	,, ,,	23·4 ,,
(*d*)	,, ,,	+0·3 cc. ,,	,, ,,	28·6 ,,

The colour developed in the Briggs modification is, as claimed by its author, independent of the degree of acidity within wide limits. We have found, however, that in estimating total phosphorus, after ignition with nitric and sulphuric acids, the addition of the Briggs molybdate solution (2 cc. of which contains 0·3 cc. conc. H_2SO_4), may bring the total acidity dangerously near to or above the upper limit of safety. We therefore prefer to add 2 cc. of a 5 % solution of ammonium molybdate alone, but take 2 cc. of the Briggs molybdate solution as usual for the standards and make up the total volume to 15 cc. in each case. For the acid ignition we use 1 cc. of a mixture of one part by volume of conc. H_2SO_4 to two parts conc. HNO_3 and carry out the ignition in 50 cc. round-bottomed flasks of resistance glass. Silica pebbles are not necessary as there is no danger of loss by spirting.

Using standard phosphate solutions, very low results were at first frequently obtained, and losses by volatilisation or by formation of pyrophosphoric acid as suggested by Baumann [1922], were suspected to be the cause. Experiments failed to confirm these suspicions, and the losses were finally traced to over ignition, resulting in some of the phosphoric acid being taken up by the glass. The errors were entirely avoided by using more moderate temperatures and stopping the ignition as soon as the white fumes of SO_3 appeared.

Error caused by trichloroacetic acid.

That trichloroacetic acid may be a source of error in the Briggs method was first suspected in the course of experiments reported by Kay and Robison [1924], where double the usual amount of this acid happened to be present in the solutions in which the inorganic phosphate was being estimated.

Investigation showed that trichloroacetic acid produces a blue colour with the Briggs reagents in complete absence of inorganic phosphate. The colour is, however, produced very slowly, and with the customary amounts of trichloroacetic acid used for the precipitation of blood proteins the error thus caused is very small, provided that the colorimetric readings are made not more than 30 minutes after adding the reagents. The following results show the amount of the error under varying conditions and prove also that it is not due merely to the presence of traces of phosphate in the trichloroacetic acid. If this were so, the error would be the same whether the readings were made after 30 minutes or several hours. The increase is however progressive, being inconsiderable at the end of half an hour and rising to very large amounts at the end of 29 hours. This indicates that the reduction of the molybdic acid is catalytically accelerated by the trichloroacetic acid in the same way as by the phosphoric acid, though in a much lower degree[1].

The intensity of the colour developed by the trichloroacetic acid appears to vary with the amount of phosphate present.

Apparent amounts of inorganic phosphate (mg. P) estimated in presence of varying proportions of trichloroacetic acid.

Amount of phosphate added	0				0·050 mg.			0·100 mg.	
Amount of 25 % trichloroacetic acid solution added	0	·5 cc.	1 cc.	2 cc.	·5 cc.	1 cc.	2 cc.	·5 cc.	1 cc.
Read after ½ hr.	0	·002	·0025	·003	·051	·053	·058	·102	·104
1½ hrs.	0	·0035	·0045	·007	·056	·062	·070	·108	·112
3 hrs.	0	·006	·0095	·014	·075	·086	·103	·127	·144
29 hrs.	0	·039	·061	·104	·105	·143	·222	·161	·200

The possibility of errors due to this cause is the more serious because the permanence of the colour is one of the advantages of this method and may lead to the practice of taking the readings after fairly long intervals.

[1] (*June* 20th, 1924.) The source of this error has been traced to an impurity present in all samples of trichloroacetic acid that have been used. The acid was obtained from the B.D.H. and distilled at an almost constant temperature. The small amount of residue contained the whole of the impurity and gave an intense coloration with the Briggs reagents, while the distillate gave no colour at all in 4 hours. The colour-producing substance was destroyed on ignition and is therefore apparently not inorganic.

THE INCREASE OF INORGANIC PHOSPHATE IN THE BLOOD AFTER WITHDRAWAL FROM THE BODY.

The increase in the inorganic phosphate which occurs in blood after withdrawal from the body has been frequently noted. It was important to discover under what conditions this increase takes place and by what means it can be prevented. On these points answers were provided by the following experiments.

Estimations of inorganic phosphate were carried out by the Briggs method on different portions of the same whole blood after treatment in various ways as described below. Except where otherwise stated the estimations were made as soon as the trichloroacetic acid filtrates were obtained (about a quarter of an hour after precipitating the proteins) and the colours were matched 30 minutes after adding the reagents. Trichloroacetic acid is referred to as "t. acid."

Exp. A. Mixed venous and arterial blood of rabbit, containing potassium oxalate.

Exp. B. Blood from ear vein of a rabbit, containing potassium oxalate.

Treatment of blood.

		Inorganic phosphate (mg. P) per 100 cc.
Exp. A.	1. Laked with water (3 vols.) and proteins precipitated with t. acid (final conc. 2·5 %) within a few minutes of withdrawal of blood from animal	6·2
	2. Phosphate estimated in the same acid filtrate as No. 1, but after this had remained 5 hrs. at room temperature ...	6·2
	3. Laked at once; laked blood kept 3 hrs. at room temperature before adding the t. acid	8·8
	4. Unlaked blood kept 3 hrs. at room temperature, then laked, and t. acid immediately added	6·3
	5. Same as No. 4, but unlaked blood kept 6 hrs. at room temp.	6·3
Exp. B.	1. Blood laked by running it directly into a 1 % solution of t. acid (3 vols.). After 5 minutes proteins precipitated by addition of more t. acid to make final concentration 2·5 %	5·3
	2. Laked with water; t. acid added after 5 mins.	5·8
	3. Laked with water; t. acid added after 2 hrs. at room temp.	7·9
	4. Laked with water and kept at 37° for 2 hrs. before adding t. acid	9·7
	5. Laked with 1 % t. acid; the rest of the t. acid added after 2 hrs. at room temp.	5·3
	6. Laked with 1 % t. acid and kept at 37° for 2 hrs. before adding the rest of the t. acid	5·3

These results show that the amount of inorganic phosphate in whole blood rapidly increases after the blood has been laked with distilled water. In laked blood kept at room temperature the increase amounted to 10 % in five minutes and 50 % in two hours. At 37° the increase was considerably greater. No increase at all occurred even in two hours at 37° in presence of 1 % trichloroacetic acid, which indicates that the change is due to enzymic hydrolysis.

In unlaked blood kept at room temperature only a very slight increase occurred in six hours, but experiments reported by Kay and Robison [1924] show that when unlaked blood is kept for five hours at 37° the amount of inorganic phosphate in the plasma is increased by about 20 %.

The practical conclusion to be drawn from these facts is that if in estimating inorganic phosphates in blood even a few minutes are allowed to elapse between laking and precipitation of proteins the results obtained will be too high. Wherever possible we now pipette the whole blood directly into three or four volumes of 1 % trichloroacetic acid.

These changes and the nature of the ester and of the enzyme concerned are being further investigated.

THE INCREASE OF INORGANIC PHOSPHATE IN BLOOD AS A RESULT OF ETHER ANAESTHESIA AND SURGICAL SHOCK.

As stated elsewhere [Kay and Robison, 1924], considerable differences were observed in the amount of inorganic phosphate in blood taken from the ear vein of a rabbit and that taken half-an-hour later from the carotid after anaesthesia. We suspected that this difference might be partly or wholly due to the effect of shock, and carried out some experiments to test this, using the precautions mentioned above.

	Description of blood	Inorganic phosphate (mg. P) in 100 cc. whole blood	Total phosphorus (mg. P) in 100 cc. whole blood
1 *a.*	Human, venous, from med. basilic vein	2·56	—
b.	Human capillary from finger of same subject taken immediately after 1 *a*	2·60	—
2 *a.*	Rabbit, venous (ear vein)	3·03	—
b.	„ „ after 10 mins. ether anaesthesia	3·37	—
3 *a.*	Rabbit, venous (ear vein)	2·62	25·9
b.	„ „ after 15–20 mins. ether anaesthesia	3·90	28·0
4 *a.*	Rabbit, from ear vein	2·86	25·4
b.	„ „ left ventricle	2·86	26·0
c.	„ „ ear vein ½ hr. after cardiac punct.	3·95	27·2
d.	„ mixed blood from carotid and jugular taken at death shortly after *c*.	5·52	30·0

These results show that the amount of inorganic phosphate in whole blood is increased after ether anaesthesia and also as the result of shock consequent upon cardiac puncture. As to the cause of this increase we are not prepared at present to give an opinion. In experiments 3 and 4 the amount of total phosphorus was also increased, which differs from the result noted by Kay and Robison [1924]. The possibility of differences existing in blood before and after passage through the muscles is not of course excluded, though the single experiment (No. 1 of the above series) suggests that such is not the case.

From what has been stated above it will be seen that there are many pitfalls in the determination of phosphorus compounds in blood, and that though under normal conditions the consequent errors may not be very large they may be very considerably increased if the conditions are but slightly changed.

REFERENCES.

Baumann (1922). *Proc. Soc. Exp. Biol. Med.* **20**, 171.
Bell and Doisy (1920). *J. Biol. Chem.* **44**, 55.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Kay and Robison (1924). *Biochem. J.* **18**, 755.