CIV. THE ESTIMATION OF PHOSPHORUS COMPOUNDS IN BLOOD.

By ROBERT VINER STANFORD
AND ARNOLD HERBERT MAURICE WHEATLEY.

From the Chemical Laboratory, Cardiff City Mental Hospital.

(Received June 15th, 1925.)

A CRITICAL perusal of the literature on this subject will show that in few cases have the proposers of methods adduced any proof of their accuracy. As we were in need of a convenient method of *known* probable error we subjected the question to a rather prolonged investigation, and were able finally to establish a procedure by means of which total, inorganic, lipin¹ and (if desired) "acid-soluble" phosphorus may be conveniently estimated in blood, plasma or corpuscles with a probable error not exceeding $\pm 5\%$.

Only two of the published methods appear to be sufficiently simple and convenient for routine use, namely, the colorimetric method described in its first form by Bell and Doisy [1920], and later modified by Briggs [1922, 1924], and the nephelometric method of Bloor [1918].

Numerous preliminary experiments with Bloor's method convinced us that the inconvenience and uncertainty of the process made it most undesirable if large numbers of analyses were to be done quickly.

Bell and Doisy's method [1920] as modified by Briggs [1922] has been used fairly extensively, but no satisfactory evidence as to its reliability has ever been furnished. Briggs [1922] talks of a "non-fading colour for comparison, the proportionality of which is exact over a wide range." In his second paper [1924] he merely states that certain suggested changes leave "the proportionality to inorganic phosphorus unimpaired." Martland and Robison [1924] remark that "there are many pitfalls in the determination of phosphorus compounds in blood," but they do not seem to suspect any lack of proportionality between the colour developed and the amount of phosphate present.

We set out to see how far Briggs' method satisfied the following criteria:

- (1) Correct estimation of known amounts of phosphates in pure aqueous solutions.
- (2) Agreements of duplicate analyses of the same blood or blood products.
- (3) Correct recovery of known amounts of phosphate added to blood or blood products.

The method depends on the fact that the phosphomolybdic acid produced when molybdic acid is added to a solution containing phosphate is reduced with formation of a blue colour by many reducing agents, the one used by Bell

¹ "Lipin" is used throughout in place of the old term "lipoid."

and Doisy and by Briggs being quinol (hydroquinone). Since the coloration with the small amounts of inorganic phosphorus normally present in blood may be too pale for good colorimetric comparison, we tried various other reducing agents, such as amidol and metol. With metol more intense colorations can be obtained, but unfortunately they are so much less stable that we returned to quinol as reducer.

Degree of acidity. The degree of acidity of the complete mixture at the time colour begins to develop has an important influence on the depth of coloration produced by any given amount of phosphate.

With gradually increasing concentration of acid (sulphuric acid) in the final solution in which colour develops, the depth of coloration produced increases to a maximum and then decreases. Further, in the neighbourhood of the maximum the coloration is less stable than when it is paler than the maximum. The instability is manifested by a gradually increasing depth of colour terminating sooner or later in the appearance of inky-blue streaks spreading through the liquid. This change, which in the less stable solutions may become apparent in little more than an hour, obviously indicates a general reduction of the molybdic acid, but the cause of it is obscure. It always begins at the surface of the liquid, but it is not due to contact with atmospheric oxygen or to the absence of sulphur dioxide from the solution.

From these observations it is clear that it is necessary to compromise between the degree of acidity giving the maximum coloration and the degree of acidity which gives the maximum stability, because at maximum stability the coloration is too pale for satisfactory colorimetric comparison. From the behaviour of our series of mixtures we consider that it is best so to arrange matters that the final solution in which the colour is developing is $N/2$ in respect of the sulphuric acid in it.

The question of acidity must also be considered from another aspect. The phosphorus of a blood product must be brought into the form of inorganic phosphate for its actual estimation. In the case of the total phosphorus and the lipin phosphorus this is effected by digestion with sulphuric and nitric acids, and the acidity of the final solution is due solely to the sulphuric acid contained in it. But in the case of the inorganic phosphorus, the blood, plasma, or corpuscles are first precipitated with trichloroacetic acid to remove proteins, and the filtrates contain considerable quantities of that acid. Martland and Robison mention that trichloroacetic acid contains an impurity (removable by distillation) which itself slowly develops a coloration with molybdate and quinol. This we can confirm, but we cannot confirm their statement that trichloroacetic acid so purified does not affect the colour development in a mixture containing phosphate, molybdate, quinol, etc. On the contrary, it is necessary not only to balance the sulphuric acid content of standard and unknown, when the solution is a digestion product, but also to balance the trichloroacetic acid contents when the unknown is a filtrate. The balancing is best done by titrating an aliquot part of the diluted digestion

product or filtrate (with sodium hydroxide and phenolphthalein) and adding the amount of sulphuric acid or of trichloroacetic acid so determined to the standard; but using the quantities detailed below in *Experimental Data* we found that the quantity of sulphuric acid to be added to the standard for a digestion product was always such as to make the final solution in which the colour develops very nearly $N/2$ in respect of this acid, and the amount of trichloroacetic acid to be added to a filtrate standard such as to make it $N/10$ in the case of a whole blood filtrate. In the case of the plasma rather more and in the case of the corpuscles rather less free trichloroacetic acid remains in the filtrate. Hence it is necessary to use the three different standards mentioned in *Experimental Data* for filtrates from whole blood, plasma and corpuscles respectively.

It will be seen that, when a product for comparison is obtained by digestion, it and its standard are $N/2$ as regards sulphuric acid, whilst in the case of a filtrate the acidity is $N/2$ in regard to sulphuric acid plus about (in all three cases) $N/10$ as regards trichloroacetic acid. As we have purposely chosen to work in a fairly stable range of the scale of acidity this difference between $N/2$ in the one case and $N/2$ plus about $N/10$ in the other does not matter, provided the appropriate standards are used. This means that in the case of a digestion product the requisite quantity of sulphuric acid must be added to the standard, and in the case of a filtrate the requisite quantities of sulphuric acid *and* of trichloroacetic acid must be added to the standard. In the latter case the same quantity of sulphuric acid must, of course, be added to the filtrate also. The most convenient way of doing this is to employ for the filtrate estimations a 5 % molybdate solution to which the requisite proportion of sulphuric acid has been added, and to use for the digestion products a neutral molybdate solution, and add to the "digestion" standard the sulphuric acid beforehand.

Proportionality of colour produced to phosphate present. It has already been mentioned that no experimental data seem to have been published on this point.

We consider that the estimations about to be referred to prove that unknown and standard should not differ by more than about 30 % if the results are to be free from uncertainty.

Preliminary experiments (four in each case), using Briggs' standard and our own, which is twice as strong, gave a maximum error of 3 % with concentration differences up to 100 %, but later experiments on blood products indicated that this agreement was fortuitous. Thirteen pairs of estimations were then made, the first of each pair employing our usual standard (except in four cases, where Briggs' standard was used) and the second of each pair using a standard nearly of the concentration of the unknown. In the first of each pair the unknown was about two and a half times the strength of the standard, and the errors ranged from + 14 % to + 7 %. In the second of each pair, where unknown and standard did not differ by more than about 10 %,

the errors ranged from 0 % to - 3 %. Similar results were obtained in the recovery of inorganic phosphate added to blood filtrates.

Finally, we compared with our standard a series ranging in concentration from two-thirds of the standard by six steps up to one and a half times the standard. This series was repeated five times. The errors of all thirty estimations lay between - 2 % and + 5 %.

Our experiments on recovery of phosphate from blood products (compare next paragraph) show that the permissible difference of concentration is then frequently smaller than in the case of the pure phosphate solution, and approximates to the 25 % difference mentioned by Myers and Shevsky [1921] and confirmed by Randles and Knudson [1922] as being the limit for the original Bell and Doisy method.

Recovery of phosphate added to whole blood. Inorganic phosphate added to whole blood is quantitatively recovered (maximum observed error 5 %) if the blood plus phosphate is digested, and the added phosphate reckoned by deduction of the *total* phosphorus of the blood. If, however, the blood after addition of phosphate is precipitated with trichloroacetic acid and the added phosphate is estimated by deduction of the inorganic phosphate of the plain blood, the results are up to 13 % too high, although duplicate analyses *using the same blood* agreed within 2 % in every instance. This is the only case in which quantitative recovery (within 5 %) of added phosphate cannot be obtained. The effect is possibly due to an alteration in the p_H of the plasma caused by the added phosphate [compare Martland, 1925].

Recovery of phosphate added to plasma. In eight experiments the errors ranged from + 1 % to + 4 %.

Agreement of duplicate analyses of inorganic phosphorus in whole blood. The bloods were laked with 1 % trichloroacetic acid as described by Martland and Robison. Fifteen samples were dealt with, from each of which two separate filtrates were made. The differences between duplicates ranged from 0 % to + 6 % (in one case).

General conclusions as to the estimation of inorganic phosphate.

We think it follows from the experiments above described that small quantities of inorganic phosphate may be recovered from pure phosphate solutions, from plasma or plasma filtrate, and from blood filtrate with a probable error of not more than 5 % provided that:

- (1) Approximately equal concentrations of the colour-producing reagents and approximately equal temperatures are maintained.
- (2) The specified degree of acidity exists in the solutions at the time the colour is developing and that it is the same in both solutions.
- (3) The phosphate concentrations of unknown and standard do not differ by more than about 30 %.
- (4) Where trichloroacetic acid is present in the unknown it is balanced by an equal amount in the standard.

Inorganic phosphate added to *whole blood* before precipitation is not quantitatively recovered within this limit, probably owing to changes caused by the added phosphate itself, but the agreement of duplicate analyses of any particular blood, with or without added phosphate, is as good as that observed with pure phosphate solutions, plasma, or with blood or plasma filtrates.

Improved digestion process for total and lipin phosphorus.

For the estimation of total phosphorus, lipin phosphorus and "acid soluble" phosphorus (*i.e.* total phosphorus of a filtrate) the phosphorus must be brought into the form of inorganic phosphate. This has been usually effected by means of digestion with a mixture of concentrated sulphuric and nitric acids (H_2SO_4 one part, HNO_3 two parts), but many references are to be found in the literature to difficulties which have been met with in carrying out such digestions. These troubles arise chiefly from the fact that whilst it is necessary to destroy all the organic matter and to obtain a final product absolutely free from nitric acid—these considerations require a fairly high temperature—yet if the temperature is too high not only is sulphuric acid lost (which upsets the final degree of acidity) but phosphoric acid may volatilise with it, as Baumann [1924] has shown. For this reason Baumann proposes to replace the nitric acid by concentrated hydrogen peroxide. We have not tried this, as with the procedure about to be described the nitric acid digestions need give no trouble.

The heat applied should be so regulated right from the start that it is just sufficient, when all the water, etc. has boiled off, to keep the remaining small quantity of sulphuric acid just at or below its boiling-point. Using the ordinary micro-burners which have been generally recommended it is very difficult to hit upon or to maintain precisely this measure of heat. In our experience to run two digestions simultaneously requires the continuous attention of a skilled person for a considerable time, and even then losses from overheating occasionally happen.

Electrical heater. This uncertainty and error and the tedious personal attention can be entirely eliminated by the use of a suitable electrical heater. With such a heater it is also possible to carry out any number of digestions simultaneously.

The instrument was improvised from the heating elements of an ordinary electric radiator which is on the market¹, and which by a fortunate chance might have been designed for the purpose.

The heating elements referred to consist of porcelain troughs $5\frac{1}{2}$ inches long and $1\frac{1}{8}$ inches wide divided by lateral porcelain partitions (the whole is in one piece) into six small square compartments each measuring about $\frac{3}{4}$ inch square (internal) at the top. The partitions are pierced with holes

¹ The radiator in question is known as the Revo, and is made by the Cable Accessories Company, Tividale, Staffs. A set of four heating elements each containing six compartments can be purchased for 12s.

through which the spiral resistance wire passes. The wire is in this way suspended about the middle of the trough. For the purpose of these digestions it is necessary that the bottoms of the pyrex tubes should be just in contact with the hot spiral, but it will be found that in the case of the usual 250×25 mm. tubes the squares are slightly too small to permit of this. The spiral is therefore removed and the internal edges of the square cautiously chamfered off (by holding them against a carborundum stick rotated in the lathe) until, on trial, the tube is found just to touch an inserted twist drill or similar object of the same diameter as the spiral. The heating element thus modified is mounted on a sheet of uralite which is separated by two half-inch pieces of wood from a light wooden base-board. The heater is then placed between the end pieces of a light wooden test-tube rack about 10 inches high, the top of which is bored with $1\frac{1}{8}$ inch holes in positions corresponding with the square compartments of the porcelain below. The instrument is connected in series with a sliding rheostat of suitable resistance.

When all the more volatile matter has boiled off from a digestion there is left about 0.3 cc. or 0.4 cc. of sulphuric acid. It is necessary to find by trial that setting of the rheostat which will give the heat required to keep this small quantity of acid just in the neighbourhood of its boiling-point, so that the production of only an occasional bubble of gas is to be noticed in the liquid. Once this point has been found, and given a reasonably constant voltage, the apparatus needs no further attention and is merely switched on every time a new batch of tubes is introduced.

If the degree of heat applied is that specified above there will usually still be nitric acid present when the transitory production of dense fumes indicates the departure of the last traces of water, for the fumes will be brown. If the liquid is colourless, it is only necessary to continue the heating until no brown fumes are to be seen, and then add the sugar solution mentioned below. The liquid may, however, still be brown. In this case it is of no use to continue heating after the brown fumes have disappeared. The current should be cut off, and when the tubes are nearly cold one or more drops of concentrated nitric acid should be added and the heating continued.

Addition of sugar. If the process is conducted as described no loss of sulphuric acid or phosphoric acid by volatilisation can occur, because after the water has disappeared all the vapours condense within the tube; but there is another possibility that must be guarded against. Bloor [1918] obtained low results if the digestion was stopped at this stage, and ascribed them to the presence of some combination of phosphoric and nitric acids which did not react with strychnine molybdate. Martland and Robison [1924] sometimes obtained low results, which they considered due to absorption of phosphoric acid by the glass in consequence of overheating. In any case, nitric acid or its decomposition products frequently remain in the product even after prolonged heating subsequent to the disappearance of brown fumes, for they can be smelt in the cold digestion product. Whether they bind any phosphoric acid or not,

these nitrogen compounds must be removed, because they upset the colorimetry. When the digestion product is colourless and no more brown fumes are visible we therefore (following Bloor) add as many drops of 1 % cane sugar as will cause a distinct browning of the partially cooled but still hot liquid. The current is then switched on and the heating continued until the liquid is colourless again.

EXPERIMENTAL DATA.

We give below the quantities to be taken for the various blood phosphorus estimations in accordance with the results which have been described above. The actual quantities mentioned are based on the obtaining of 15 cc. of the final blue solution of a depth of colour suitable for comparison in a 25 mm. cell in the dilution colorimeter [Stanford, 1923]. Should any other final volume be desired it is important to note that the concentration of *all* the substances present in that final solution in which the colour is developing must be that here given.

1. *Reagents*¹.

(a) Acid molybdate (for inorganic phosphorus in blood, plasma or corpuscles).

Dissolve 25 g. of ammonium molybdate in 300 cc. of water, then add 75 cc. of concentrated sulphuric acid previously diluted with 125 cc. of water.

(b) Neutral molybdate (for total, lipin and "acid soluble" phosphorus) is a filtered 5 % solution of ammonium molybdate.

(c) Quinol 1 % solution.

(d) Sodium sulphite 20 % solution.

2. *Standards for inorganic phosphate.*

	(a) For whole blood	(b) For plasma	(c) For corpuscles
KH ₂ PO ₄ (1 cc. $\equiv$ 6.0 cmgm. P)	72.0 cc.	72.0 cc.	72.0 cc.
CCl ₃ .COOH (20 % solution)	74.4	91.2	57.6
Water	357.6	340.8	374.4
	504.0	504.0	504.0

3. *Estimation of inorganic phosphates.*

Mix 20 volumes of blood, plasma, or corpuscles with 60 volumes of 1 % trichloroacetic acid, then add 17 volumes of 30 % trichloroacetic acid and 3 volumes of water. Shake vigorously, and allow to stand for a few minutes before filtering. To 10 cc. of the filtrate add 0.5 cc. of a KH₂PO₄ solution containing 8 cmgm. of P per cc. (This 4.0 cmgm. is of course to be deducted when calculating the result.) To the 10.5 cc. of solution so obtained 1.5 cc. each of *acid* molybdate, quinol and sulphite are successively added, making a total volume of 15 cc. To 10.5 cc. of the appropriate standard (see above,

¹ The abbreviation "cmgm." stands for "hundredth of a milligram."

2 (a), (b) or (c)) 1.5 cc. each of *acid* molybdate, quinol and sulphite are added. The additions of molybdate, etc. to the two solutions should be as nearly simultaneous as possible, and the colorimetric comparison should be made after 30 minutes.

4. *Standard for total, lipin and "acid soluble" phosphorus.*

KH_2PO_4 (1 cc. $\equiv$ 6.0 mgm. P) ...	...	72.0 cc.
H_2SO_4 (10N) ...	...	36.0
Water ...	...	396.0
		<hr/> 504.0

5. *Estimation of total phosphorus.*

Take of whole blood 0.4 cc., or of plasma 1.5 cc., or of corpuscles 0.25 cc. (previously laked with an equal volume of warm water), and digest with 1 cc. of a mixture of concentrated sulphuric acid (1 part) and concentrated nitric acid (2 parts), adding three small pieces of quartz to prevent bumping. After digestion make up to 15 cc. in a graduated tube. Of each digestion product take 10 cc., add 0.5 cc. of water, followed by 1.5 cc. each of *neutral* molybdate, quinol and sulphite. The standard solution simultaneously set up contains 10.5 cc. of the standard for total, etc. phosphorus given above, paragraph 4, and 1.5 cc. each of *neutral* molybdate, quinol and sulphite.

6. *Estimation of lipin phosphorus.*

Take of whole blood 1.5 cc., or of plasma 2.0 cc., or of corpuscles 1.0 cc. (previously laked with an equal volume of warm water). In each case add drop by drop to about 20 cc. of a mixture of alcohol (redistilled) 3 parts and ether (redistilled) 1 part. Heat to boiling in a water-bath, cool, filter through a small paper into a pyrex tube (250 $\times$ 25 mm.) and boil off the solvent in the water-bath. The blood product is repeatedly washed with small quantities of the extraction mixture, the washings being filtered into the pyrex tube and boiled off. The tube is finally left in boiling water for a few minutes. The acid digestion mixture (1 cc.) is then added and the subsequent procedure is the same as that for total phosphorus.

SUMMARY.

The conditions are described which are necessary for obtaining accurate results in the estimation of inorganic phosphate by the colorimetric method.

The process of digestion with acids used for bringing total P or lipin P into the form of inorganic phosphate has also been studied, and an electrical heating device is described.

Methods are given in detail for the estimation of inorganic, total, lipin and "acid soluble" P in blood, plasma and corpuscles, and evidence is presented that the results are accurate to within a probable error of 5 % of the amount of phosphorus estimated.

We are indebted to our colleagues, Dr J. Walker and Dr S. Grossman, for the collection of the very numerous samples required for this investigation.

A part of the expenses of the work has been borne by the Medical Research Council, to whom our thanks are due.

REFERENCES.

- Baumann (1924). *J. Biol. Chem.* **59**, 667.
Bell and Doisy (1920). *J. Biol. Chem.* **44**, 55.
Bloor (1918). *J. Biol. Chem.* **36**, 33.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
— (1924). *J. Biol. Chem.* **59**, 255.
Martland (1925). *Biochem. J.* **19**, 117.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
Myers and Shevsky (1921). (Cited by Anderson (1923), *Biochem. J.* **17**, 44.)
Randles and Knudson (1922). *J. Biol. Chem.* **53**, 55.
Stanford (1923). *Biochem. J.* **17**, 839.