THE PRESERVATION OF BOVINE BLOOD FOR THE DETERMINATION OF INORGANIC PHOSPHATE IN THE DIAGNOSIS OF APHOSPHOROSIS.

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SUMMARY.

An improved method of preserving and processing bovine blood for the determination of the level of inorganic phosphate is described.

The incomplete precipitation of protein in dilute trichloracetic acid, which is noted particularly at high atmospheric temperatures and which interferes with colorimetric estimation, may be avoided by adding fullers earth and trichloracetic acid to the filtrate prepared for treatment for colorimetric estimation.

The low final concentration of trichloracetic acid attainable by this procedure permits the storage of the blood for several days without appreciable hydrolysis of organic P. compounds to inorganic P.

INTRODUCTION.

The observation of Theiler, Green and Du Toit (1927) that a fall in the inorganic phosphorus (denoted by P.) level in bovine blood preceded any outward clinical symptoms of aphosphorosis provided a useful means of diagnosing aphosphorosis in its early stages.

The method used by these workers for the determination of inorganic P. was precipitation of blood protein with 5% trichloracetic acid, followed by colorimetric estimation of inorganic P. as reduced phosphomolybdate in the trichloracetic acid filtrate. Because inorganic P. is liberated from organic P. compounds with time in whole blood and in trichloracetic acid filtrates of whole blood, the method was of limited usefulness in cases where blood samples took several days to reach the analyst.

Malan (1930) reported that hydrolysis of organic P. compounds to inorganic P. proceeds more slowly in dilute solutions of trichloracetic acid. A final concentration of 2·5% trichloracetic acid in the filtrate enabled blood to be stored for at least eight days and allowed a reasonably accurate diagnosis of aphosphorosis to be made.

OBSERVATIONS.

Attempts by the author to confirm Malan's procedure were unsuccessful, due to cloudiness of the developed, reduced phosphomolybdate blue making matching of colours difficult and inaccurate. This cloudiness, which is due to unprecipitated protein, is more marked at high temperatures. When inorganic P. is determined on blood which has stood for two or three days at high

atmospheric temperatures, protein is precipitated by the molybdate reagent, and appreciable quantities of the reduced phosphomolybdate are adsorbed on the surface of the precipitate. The nature of the unprecipitated protein in dilute trichloracetic acid is changed on standing, for the precipitation cannot be completed by adding further trichloracetic acid.

In attempts to overcome the difficulty caused by cloudiness, the author found that cloudiness in the developed solution may be prevented by adding a small quantity of fullers earth and trichloracetic acid to the filtrate immediately before adding the analytical reagents. This procedure permits a final concentration of 1·5% trichloracetic acid. At this concentration, the rate of liberation of inorganic P. from organic P. compounds is very slow.

The addition of isotonic saline to the trichloracetic acid preservative facilitates the subsequent filtration of the protein mass.

An indication of the suitability of the method for blood which has been stored for some time is given by the fact that in 18 samples of blood processed after storage at 18°C. for two weeks, the average increase in inorganic P. was 0·15 mg.P. per 100 ml. blood, with a maximum increase of 0·3 mg.P. in two samples (Table 1). It is unlikely that under field conditions the period required for transport and storage of blood samples would be as long as 14 days. In most cases accurate determination may be made three to four days after collection and processing of the blood sample. As might be expected, the hydrolysis proceeds more readily at high temperatures. At 37°C. the same average increase was observed in one week. No measurable hydrolysis occurs in refrigerated samples for the same period.

Table 1.

PHOSPHORUS LEVELS IN BLOOD SAMPLES PRESERVED IN 1·5% TRICHLORACETIC ACID (mg. P. per 100 ml. of blood)

No.	Initial inorganic P. levels.	Inorganic P. levels after 14 days at 18°C.	Increase in inorganic P. levels.	Hydrolysable P. ester.
1	5·1	5·3	0·2	1·7
2	3·8	3·9	0·1	1·4
3	4·8	4·9	0·1	1·5
4	2·2	2·2	0	1·0
5	2·6	2·6	0	1·2
6	3·5	3·6	0·1	1·2
7	2·8	2·9	0·1	1·3
8	3·7	3·7	0	1·3
9	6·2	6·4	0·2	1·7
10	4·3	4·5	0·2	1·6
11	4·4	4·6	0·2	1·6
12	4·8	5·0	0·2	1·5
13	3·6	3·6	0	1·4
14	4·0	4·2	0·2	1·6
15	5·3	5·6	0·3	2·0
16	4·4	4·6	0·2	1·5
17	4·1	4·3	0·2	1·5
18	5·0	5·3	0·3	1·8

Malan (1930) observed that the hydrolysis of phosphorus esters in a fixed concentration of trichloracetic acid varies irregularly in blood from different animals of the same species although he did not report the temperatures at which individual samples were kept. He postulated that the quantity of the organic acid soluble P. present was a contributing factor to the variability noted but did not attempt to confirm this statement.

This aspect was examined and the conclusions reached are summarized below:—

(i) The total organic acid soluble P. can be differentiated into a hydrolysable and an unhydrolysable fraction. The hydrolysable fraction was determined by heating 10 ml. of the filtrate from 10 ml. of blood in 40 ml. of 8% trichloracetic acid with 1 ml. of 20% H_2SO_4 for one hour at 100°C. After one hour there was no further increase in inorganic P. In 20 blood samples, the concentration of the hydrolysable P. ester fraction varied between 30% and 60% of the total P. ester.

(ii.) The rate of hydrolysis in blood in a fixed concentration of trichloracetic acid at constant temperature tends to increase as the concentration of hydrolysable P. ester increases.

(iii) Low levels of inorganic P. tend to be associated with low levels of hydrolysable P. ester.

From these observations, it is evident that the hydrolysis of P. esters generally proceeds more slowly in blood with low levels of inorganic P. In the 18 blood samples mentioned above, the average concentration of hydrolysable P. ester corresponded to 1.5 mg.P. per 100 ml. of blood (see Table 1). The two samples with the maximum increases of 0.3 mg.P. in two weeks at 18°C. coincided with the highest concentration of hydrolysable P. ester (1.8 mg. and 2.0 mg.P. per 100 ml. blood) and normal inorganic P. levels of 5.0 mg. and 5.3 mg.P. per 100 ml. blood.

PROCEDURE.

For the preservation of blood for inorganic phosphate determination, 2 oz. bottles containing 35 ml. of 2.0% trichloracetic acid and 0.9% NaCl are made up for use in the field. (At present there is some doubt as to the keeping properties of trichloracetic acid, which gives a free chloride test on standing in dilute solution. Liberated HCl would increase the [H]+, which may increase the hydrolysis of P. esters. However, from preliminary work, the above solution is considered sufficiently stable for probably two or three months.)

To the prepared bottles, 10 ml. of blood containing not more than 0.1 g. sodium citrate is added and the contents shaken.

The samples are returned to the laboratory, and immediately prior to analysis 5 ml. of 20% trichloracetic acid is added and the contents mixed. Approximately 0.5 g. of fullers earth is then added and the contents shaken and allowed to stand for at least two minutes before filtering through a No. 2 Whatman filter paper (phosphate free).

To 10 ml. of the clear filtrate is added 4.5 ml. (from a 10 ml. measuring cylinder or burette) of the following reagents mixed in equal proportions by volume immediately before use:—

(i) Brigg's reagent: 25 g. ammonium molybdate is dissolved in water with warming and made to 200 ml. This is mixed when cold with a cooled solution of 75 ml. of concentrated H_2SO_4 diluted to 300 ml. of water.

(ii) 20% aqueous sodium sulphite.

(iii) 1% aqueous hydroquinone.

The volume is made to 20 ml. and compared in a visual colorimeter after 30 minutes, but not more than 45 minutes, against 2 ml. of standard phosphate solution containing 50 μg.P. per ml.

SELECTION OF FULLERS EARTH.

The classification and physical properties of eight different batches of fullers earth and their relative capacities for adsorbing protein are given in Table 2. The blanks of 0.5 g. of these samples are expressed as mg.%P. These blanks refer to the calculated error in mg.P. per 100 ml. of blood when the above procedure is used.

Table 2.

EXAMINATION OF SAMPLES OF FULLERS EARTH.

Sample.	Classification.	Colour.	Texture.	Relative Adsorption.	Blank Mg%. P.
1	B.D.H. for adsorption ..	Grey ..	Fine powder	++	1·2
2	B.D.H. for adsorption ..	Red-brown ..	Fine powder	+	0·2
3	B.D.H. for adsorption ..	Grey-white ..	Granular ..	+	5·0
4	B.D.H. for adsorption ..	Brown ..	Lump ..	++	0·05
5	B.D.H. for adsorption ..	Brown ..	Fine powder	++	0·10
6	B.D.H. for adsorption ..	Brown ..	Fine powder	++	0·10
7	B.D.H. for adsorption ..	White ..	Fine powder	—	0·05
8	D.B.H. for adsorption ..	White ..	Fine powder	—	0·40

The batches of fullers earth with good adsorptive properties and with low blanks (<0.1 mg.%P.) could be used directly by approximating without weighing the 0.5 g. fullers earth and subtracting this blank in the determination of inorganic P., without significant error.

While the selection of fullers earth with the most desirable properties might readily be achieved by prior testing of several samples, the following method for eliminating this blank without loss in adsorptive properties was devised.

200 g. of fullers earth is stirred into a uniform paste with 250 ml. of concentrated H_2SO_4 (S.G. 1.84) and immediately poured into 3–4 litres of water. The fullers earth is washed four times with tap water by decantation and finally with distilled water. The product is then filtered through a buchner funnel, dried at 100°C. and ground to a fine powder.

It is recommended that the fullers earth to be used for adsorption, whether directly or treated, should be examined for the following characteristics under precisely the conditions for the analysis of blood.

The blank, if any, on the fullers earth should be:

(i) Proportional to the amount of fuller's earth used.

(ii) Independent of the concentration of P. to be determined.

(iii) Independent of the time, within reasonable limits, in which the fullers earth is in contact with the trichloracetic acid.

(iv) Constant for small changes in the trichloracetic acid concentration.

In addition, the rate of development of reduced phosphomolybdate in a standard P. solution treated with fullers earth should be the same as that of an untreated P. standard. A curve relating the development of colour to time may be determined in a photoelectric colorimeter at 595 mμ against a blank of the reagents at 10, 20, 30 and 40 minutes development time.

All samples of fullers earth examined conformed to the above conditions, with the exception of the treated sample, which gave a very slight asymmetric curve for the development of colour. The difference was, however, insignificant at 45 minutes development time.

The minimum amount of fullers earth required to clarify blood filtrates under the most unfavourable conditions of storage anticipated should be determined.

DISCUSSION.

In the diagnosis of aphosphorosis in cattle, a sufficiently good assessment of the phosphate status of the blood may be made if the error in the inorganic P. level does not exceed 0.3 mg.P. per 100 ml. blood.

The modification of Malan's method developed by the author permits a determination of inorganic P. within this margin of error, even after several days' storage at atmospheric temperature. Because hydrolysis in blood with a low inorganic P. level proceeds very slowly, processed samples of such blood may be kept for as long as two weeks and still yield reliable determinations.

REFERENCES.

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