CXXXI. DETERMINATION OF INORGANIC SULPHATE IN HUMAN BLOOD-PLASMA BY MICRO-TITRATION.

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Observations on the inorganic sulphate content of human blood and other body fluids are relatively infrequent, partly owing to the great difficulties encountered in estimating the small concentrations of inorganic sulphate present therein, but also because the sulphate ion has been regarded as pharmacologically inert and as therefore of little importance in the elucidation of physiological and pathological problems. That it is pharmacologically inert is probably true. The intravenous injection of sodium sulphate into human beings in amounts up to 3 or 4 g. certainly has no detectable effects save an increase in the amount of sulphate excreted in the urine[2], and Sollman [1926] states that much larger amounts may be given without demonstrable effects.

There are however certain problems in the solution of which a knowledge of the blood sulphate would be of considerable value. Peters and his associates [1929] have called attention to the probable rôle of sulphate in accounting in part for the "undetermined acid" combining with base in cases of nephritis with nitrogen retention and have made a plea for further investigation of its behaviour in the organism.

The suggestion that sulphate, being apparently merely filtered off by the renal glomeruli and not again re-absorbed in the tubules, could be used as a measure of the volume of glomerular filtrate [White, 1923; Rehberg, 1926; Sager, 1930; Poulsson, 1930], has greatly increased the need for a satisfactory method of estimation of inorganic sulphate in small quantities of blood-plasma or serum. Rehberg considered the estimation too difficult in plasma. White and also Sager, simplified the problem by artificially increasing the blood sulphate concentration to more easily estimated levels, whilst Poulsson contented himself with a rough estimate of the probable concentration in the blood during his experiments.

Numerous methods for plasma inorganic sulphate estimation have from time to time been described. The more recent of these may be classified as follows.

(*a*) *Gravimetric*, Meyer-Bisch [1924], Pohorecka-Lelesz [1927], Loeb and Benedict [1927].

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[2] Personal observation, unpublished.

(*b*) *Nephelometric*, Denis [1921], Denis and Reed [1926], Chatron [1931].

(*c*) *Benzidine, by titration*, White [1923], Sager [1930], Pohorecka-Lelesz [1927], Chatron [1931], Lesure and Dunez [1929].

(*d*) *Benzidine, colorimetric*, Yoshimatsu [1926], Wakefield [1929], Hubbard [1930].

(*e*) *Sulphide formation*, Lorant [1929].

(*f*) *Miscellaneous*, Lang [1929].

Of these different methods, the gravimetric requires either a large volume of blood or very accurate weighing on a micro-balance. The nephelometric method suffers from the fact that the turbidity of the suspension of barium sulphate is very dependent on changes in the p_H and composition of the blood-filtrate. The titration of benzidine sulphate has, so far as the writer is aware, only been applied to blood in which sulphate concentration has been artificially raised, or in which it is already higher than in human blood [Pohorecka-Lelesz, 1927].

The colorimetric benzidine processes require great care in their manipulation if colour is to remain proportional to sulphate content. The colours obtained, moreover, being weak yellows and browns, are difficult to compare accurately, at least for some workers.

Reduction to a sulphide is a somewhat laborious procedure and appears to include ethereal and other sulphates in the analysis. Finally, the method of Lang, whilst giving excellent pink colours, has not, in the hands of the writer at least, given constant results with such low concentrations of sulphate as are encountered in normal human plasma.

Numerous attempts have been made by the writer to improve on one or other of these methods. A modification of the Denis and Reed nephelometric procedure and a micro-titration method based on Hubbard's technique [1930] will be described.

Nephelometric method.

Denis's claim for an accuracy of 5 % for her modified method [1926] has been fully confirmed for calf's and sheep's serum in both of which the actual inorganic sulphate concentration is five or six times as high as in human serum. Table I shows the results obtained.

Table I. *Comparison of gravimetric and Denis nephelometric methods.*

Serum used	Gravimetric S mg./100 cc.	Nephelometric S mg./100 cc.	Error (%)
Calf's serum	5·29	5·36	1
,, ,,	7·16	6·94	3
Sheep's serum	3·59	3·73	4
,, ,,	3·51	3·73	6
Calf's serum	4·77	4·58	4
,, ,, (with added S)	5·25	5·45	4
,, ,, (diluted 1 : 1)	2·38	2·40	1
Sheep's serum	3·12	3·06	2
,, ,, (with added S)	3·74	3·71	1

When however the same method was applied to human plasma or serum, the results were far less satisfactory. Denis herself stated that, if from a blood-filtrate no precipitate is obtained with barium chloride, it is necessary before concluding that no sulphate is present to determine the p_H of the solution, the optimum value for which she gives as 3·0 to 3·8. The method as described by Denis, however, takes no account of the p_H of the final filtrate, but assumes that in all ordinary cases this will be such as to give a reliable nephelometric reading, if the reagent quantities given are adhered to.

It was felt that herein lay a possible source of the discrepant results, since, if an unfavourable p_H may completely prevent the precipitation of barium sulphate, then one somewhat less unfavourable may evidently seriously affect the resultant turbidity without necessarily arousing suspicion. This was found actually to be the case, and a p_H range of from 1·8 to 2·4 was found to yield maximum and fairly constant turbidities. This is rather more acid than the p_H recommended by Denis and slightly more so than that suggested by Chatron, but the small differences may be due to variations in the gelatin stabilisers employed.

It was, however, found that even with a constant p_H, the total concentration of trichloroacetic acid present had a serious effect on the turbidity produced. This is well shown in the following example in which a solution with a sulphate content corresponding to the filtrate from a plasma with 0·65 mg. S per 100 cc. was employed.

Cc. 20 % trichloroacetic acid present in 15 cc. of filtrate	0·1	0·25	0·5	0·75	1·0	1·5
Final p_H to thymol blue	2·5	2·4	2·4	2·3	2·4	2·2
Percentage error in estimation	6	3	6	20	28	43

The presence of small quantities of trichloroacetic acid in the solution did not materially influence the result. The excess can be conveniently removed by shaking the protein-free blood-filtrate with two volumes of ether, if allowance be made for the resultant change in volume of the water phase (about 7 %). Much more consistent results are then obtained, as is shown in the following example, in which the same concentration of sulphate as above was used.

Cc. 20 % trichloroacetic acid present in 15 cc. of filtrate	0·25	1·5	1·5	1·5
Final p_H to thymol blue	2·0	2·0	2·0	2·0
			Extracted with ether	
Percentage error in estimation	1	32	1	3·5

The same effect was observed with other low concentrations of sulphate.

It is evident therefore that in order that the nephelometric method should yield even approximately constant results with human plasma, (*a*) the p_H of the solution must be controlled in every case and brought to the optimum value; (*b*) the quantity of trichloroacetic acid present in the filtrate must be small.

The presence of ether in the extracted solution appeared to have little effect on the nephelometric readings, but it is desirable to treat the standard sulphate solutions in a similar manner.

Denis's method modified in this manner was employed for some time by the writer with fairly satisfactory results but was finally abandoned in favour of the micro-titration benzidine technique. As this has proved itself to be in every way preferable to the modified nephelometric method, details of technique of the latter will not be given.

Both procedures give figures varying from 0·5 to 1·5 mg./100 cc. of S as inorganic sulphate for normal human plasma and thus agree with the now generally accepted values.

Micro-titration benzidine method.

This is a modification of the method advocated by Hubbard [1930] and indeed follows his procedure in detail as far as the production of the washed benzidine sulphate precipitate. This Hubbard estimated colorimetrically, but estimation by direct titration has been found more reliable for low sulphate concentrations. For convenience, Hubbard's procedure is described here. For further details the original paper should be consulted.

Reagents.

(*a*) 1 % benzidine in good acetone.
(*b*) 20 % sulphate-free trichloroacetic acid in water.
(*c*) Good grade acetone.
(*d*) *N*/50 CO_2-free sodium hydroxide.

Method.

To 2 to 5 cc. of serum or citrated plasma in a centrifuge tube is added an exactly equal volume of 20 % trichloroacetic acid, and the whole is thoroughly mixed with a thin glass rod. The precipitated proteins are centrifuged, leaving a clear supernatant fluid which usually requires no further filtration.

In a 10 cc. Pyrex glass centrifuge tube with sharply pointed tip are placed 5 cc. of the 1 % benzidine in acetone solution, and to this are added exactly 2 cc. of the protein-free blood-centrifugate. The tube is stoppered and kept in ice-water for an hour, or until flocculation of the precipitate begins to occur. It is centrifuged and drained for 5 minutes, the tube being inverted in a small beaker in the bottom of which is a pad of filter-paper moistened with acetone. This prevents evaporation of the acetone before drainage is complete. 10 cc. of fresh acetone are added, the precipitate is broken up with a fine glass rod and centrifuged; the tube is again drained on the pad for 5 minutes.

Titration. For this purpose the micro-burette described by Rehberg [1925] has been found eminently suitable. It should be filled with *N*/50 CO_2-free sodium hydroxide. The titration is carried out in the Pyrex centrifuge tube itself, this being surrounded by a steam-jacket. The apparatus recommended is shown in Fig. 1.

The jacket is readily made from ordinary glass. Steam enters by the side-tube from a suitable flask, and escapes with the condensed water into a

receiver placed below the bottom tube. The upper opening carries a rubber stopper bored with a hole into which the centrifuge tube fits.

Mixing of the solution during titration is effected by bubbling through it a slow stream of CO_2-free air, most conveniently obtained from two large bottles connected by a syphon and containing weak sodium hydroxide solution. The rate of bubbling is controlled by a screw-clip.

To the tube containing the washed and drained benzidine sulphate precipitate is added 0·5 cc. of distilled water. Phenolphthalein is added by dipping the tip of a fine glass rod into the indicator solution and inserting it into the centrifuge tube, where it may be allowed to remain, as it is useful for breaking up the precipitate during the titration. The centrifuge tube with its fine glass stirring rod is inserted into the steam-jacket, and the whole arranged so that the tip of the microburette just dips under the surface of the liquid in the tube. The tube for bubbling CO_2-free air is next placed in position and a gentle stream commenced.

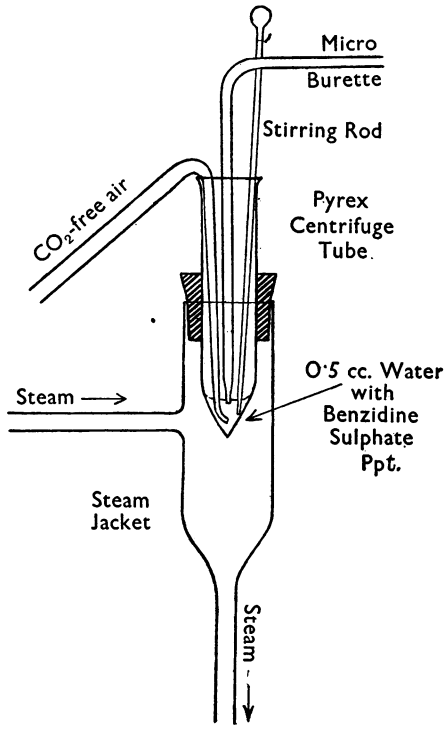

Fig. 1.

It is advisable to carry the titration as far as possible before the steam is turned on. When the pink colour due to the added alkali tends to become persistent, the steam is turned on and the titration completed as rapidly as possible at 100°. No difficulty is encountered in detecting the first appearance of a permanent pink if good lighting be arranged. Before removing the tube its sides are well washed down by turning on the air supply fully for a moment, and further alkali is added if necessary. The centrifuge tube and jacket are then immediately removed from the tip of the burette. Some haste is desirable during these later stages in order to minimise the heating of the fluid in the burette during the titration. After the tube is withdrawn cooling leads to a slight retraction of fluid from the tip of the burette. The meniscus must be brought once again to the tip by turning the control screw, before the final reading of the burette is made.

A blank titration is necessary in order to determine the amount of alkali required to produce a definite pink colour in 0·5 cc. of distilled water at 100°. This usually lies between 3·0 and 4·5 mm.^{3} of $N/50$ sodium hydroxide, and the blank must be subtracted from the titration figure.

This procedure has given more reliable results than any other tried by the writer. The figures in Table II indicate the degree of accuracy obtained.

All the determinations were performed on samples of sheep's serum which had been dialysed for 14 days against pure 0·9 % sodium chloride. It is interesting that, in the second specimen of serum, sulphate in a concentration of 0·06 mg. S per 100 cc. could be definitely detected, and produced a distinctly visible deposit of benzidine sulphate .

Table II.

Number	Added S mg./100 cc.	Theoretical S mg./100 cc.	Added citrate mg./100 cc.	Added P mg./100 cc.	mm^3 $N/50$ sodium hydroxide used (corrected)	Estimated S mg./100 cc.	(%) recovery
1	0·83	0·83	—	—	26·1	0·836	100
2	1·15	1·15	—	—	38·2	1·22	108
3	1·15	1·15	—	—	35·1	1·12	98
4	1·43	1·43	—	—	43·3	1·38	96
5	1·43	1·43	—	—	43·2	1·38	96
6	1·37	1·37	200	—	41·4	1·32	96
7	1·37	1·37	200	—	41·3	1·32	96
8	1·67	1·67	—	—	52·5	1·68	100·6
9	1·67	1·67	—	—	52·0	1·665	100
10	1·62	1·62	200	—	51·6	1·65	102
11	1·62	1·62	200	—	49·0	1·565	96·6
12	2·14	2·14	—	—	69·0	2·20	103
13	2·14	2·14	—	—	67·3	2·15	100·5
14	1·67	1·67	—	3·3	53·4	1·71	102·4
15	1·67	1·67	—	3·3	52·9	1·69	101·2
16	1·36	1·36	—	6·5	41·6	1·33	97·8
17	1·36	1·36	—	6·5	43·0	1·375	100·7
Fresh dialysed serum estimated to contain 0·06 mg. S per 100 cc.							
18	2·31	2·37	—	—	76·0	2·44	103
19	2·31	2·37	—	—	77·0	2·46	104
20	0·91	0·97	—	10·0	30·4	0·97	100
21	0·91	0·97	—	10·0	32·1	1·02	105
22	1·67	1·73	—	10·0	54·5	1·74	100·6
23	1·67	1·73	—	10·0	55·2	1·76	102
24	0·55	0·615	—	12·3	20·1	0·64	104
25	0·55	0·615	—	12·3	18·6	0·595	96·7
26	2·00	2·06	—	22·0	74·0	2·37	115
27	2·00	2·06	—	22·0	74·0	2·37	115
28	0·45	0·515	—	10·0	16·4	0·525	102
29	0·45	0·515	—	10·0	17·2	0·55	107
Third sample of dialysed serum free of sulphate.							
30	0·91	0·91	900	5·0	28·2	0·905	99·5
31	0·91	0·91	900	5·0	27·9	0·896	98·6
32	2·86	2·86	900	5·0	101·0	3·23	112
33	2·86	2·86	900	5·0	97·0	3·11	108
34	3·33	3·33	900	5·0	111·0	3·56	107
35	3·33	3·33	900	5·0	114·0	3·64	109
36	3·85	3·85	900	5·0	129·0	4·14	107·5
37	3·85	3·85	900	5·0	130·0	4·16	108

The figures show that even a large excess of citrate has no appreciable effect on the estimation.

Concentrations of phosphate up to 12 mg. P per 100 cc. of original plasma have no effect on the results, and the figures show a satisfactory agreement with the theoretical up to about 2·5 mg. S per 100 cc. but tend to be a little too

high when the sulphate concentration exceeds that value, and much phosphate is present.

SUMMARY.

Some errors in the nephelometric method of estimating inorganic sulphate in small amounts of human plasma or serum are considered, and means of overcoming them are suggested.

Details of a modified benzidine technique are given in which the more usual colorimetric estimation is replaced by micro-titration with sodium hydroxide. With the filtrate from 1 cc. of human serum or plasma this method gives results within 5 % of the theoretical.

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REFERENCES.

Chatron (1931). *Bull. Soc. Chim. Biol.* **13**, 300.
Denis (1921). *J. Biol. Chem.* **49**, 311.
—— and Reed (1926). *J. Biol. Chem.* **71**, 191.
Hubbard (1930). *J. Biol. Chem.* **88**, 663.
Lang (1929). *Biochem. Z.* **213**, 469.
Lesure and Dunez (1929). *Bull. Soc. Chim. Biol.* **10**, 879.
Loeb and Benedict (1927). *J. Clin. Invest.* **4**, 33.
Lorant (1929). *Z. physiol. Chem.* **185**, 245.
Meyer-Bisch (1924). *Biochem. Z.* **150**, 23.
Peters, Wakeman, Eisenman and Lee (1929). *J. Clin. Invest.* **6**, 517.
Pohorecka-Lelesz (1927). *Bull. Soc. Chim. Biol.* **9**, 263.
Poulsson (1930). *Z. ges. exp. Med.* **71**, 577.
Rehberg (1925). *Biochem. J.* **19**, 270.
—— (1926). *Biochem. J.* **20**, 447.
Sager (1930). *Arch. exp. Path. Pharm.* **153**, 331.
Sollmann (1926). Manual of pharmacology, p. 824. (Philadelphia.)
Wakefield (1929). *J. Biol. Chem.* **81**, 713.
White (1923). *Amer. J. Physiol.* **65**, 537.
Yoshimatsu (1926). *Tohoku J. Exp. Med.* **7**, 119.