CXXXVIII. A PRELIMINARY NOTE ON THE INORGANIC SULPHATE CONTENT OF THE BLOOD WITH A METHOD FOR ITS DETERMINATION.

BY DAVID PATON CUTHBERTSON
AND SIDNEY LIONEL TOMPSETT.

*From the Biochemical Laboratory of the Institute of Pathology
of the Royal Infirmary and University of Glasgow.*

(Received July 2nd, 1931.)

THE methods evolved for the determination of the inorganic sulphate content of the blood may be conveniently summarised as follows: (1) nephelometric, Denis [1921]; (2) volumetric, White [1923]; (3) gravimetric, Loeb and Benedict [1927]; and (4) colorimetric, Yoshimatsu [1926] and Hubbard [1927, 1930]. As the concentration of inorganic sulphate in the blood is very low, relatively large quantities of material are required for satisfactory gravimetric and volumetric analyses. It was therefore decided to investigate first the colorimetric method of Hubbard [1927, 1930]. In this method the blood-serum or blood-plasma is deproteinised with trichloroacetic acid, and the inorganic sulphate in the filtrate is then precipitated as benzidine sulphate in an acetone medium. The precipitated benzidine sulphate after solution in dilute hydrochloric acid is determined colorimetrically by the addition of hydrogen peroxide and ferric chloride, a yellow colour being produced. In their experiments with this method the present writers found that in the case of normal sera and plasma the concentration of inorganic sulphate was too low to produce a colour sufficiently intense to be of value for accurate comparison. There was, therefore, a need for a more sensitive colour reaction for benzidine.

Finally it was decided to modify the series of reactions used by Kahn and Leiboff [1928] for the determination of inorganic sulphates in urine, *viz.* diazotisation of the precipitated benzidine followed by coupling with phenol in alkaline solution. To intensify the colour the present writers have used thymol in place of phenol. The resultant colour is red.

Technique.

A solution containing 2.0071 g. benzidine hydrochloride in 500 cc. *N* HCl was prepared. Dilutions were then prepared of which 1 cc. was equivalent to 0.0025, 0.005, 0.01, 0.02, 0.04 and 0.08 mg. sulphur respectively. These dilutions were made with *N* HCl. To 2 cc. of each of these solutions 1 cc. of freshly prepared 0.1 % sodium nitrite was added. After shaking and allowing to stand 1 minute, 5 cc. of 15 % sodium hydroxide were added, and, after

thorough mixing, 5 cc. of 1 % thymol in 10 % sodium hydroxide were added to each. A red colour developed, very intense for the concentrations of benzidine chloride used. It was considered that this colour reaction would be much more suitable than the reaction used by Hubbard [1927, 1930].

Wakefield's [1929, 1] lowest standard was a dilution equivalent to 0.005 mg. sulphur, but it was found that a standard half this strength was necessary for the determination of the inorganic sulphate content of normal blood and serum. The colour obtained by coupling thymol with diazotised benzidine reached a maximum in 15 minutes and did not fade for 1 hour. The colour production was practically proportional to the amount of benzidine over this range, but in all estimations standards of varying benzidine content were prepared. In determining the inorganic sulphate content of blood, serum and plasma, all glass apparatus must be scrupulously clean and where potassium oxalate is used to prevent clotting, it must be shown to be free of all traces of sulphate.

The following technique was used for the determination of the inorganic sulphate content of blood, serum and plasma.

Reagents.

1. Acetone.
2. 20 % trichloroacetic acid in distilled water. Commercial trichloroacetic acid is purified by distillation with benzidine.
3. 0.5 % benzidine in acetone. This should be prepared fresh each day.
4. Benzidine hydrochloride standard solution.
2.0071 g. of benzidine hydrochloride are dissolved in 500 cc. *N* HCl. 1 cc. of this solution is equivalent to 0.5 mg. sulphur and is used as a stock solution. More dilute solutions for use as standards for the determination of inorganic sulphate in blood, being equivalent per cc. to 0.0025 mg., 0.005 mg., 0.01 mg., 0.02 mg., 0.04 mg. and 0.08 mg. sulphur, are prepared by diluting the stock solution with *N* HCl.
5. 0.1 % sodium nitrite solution—prepared fresh each day.
6. 15 % sodium hydroxide solution.
7. 1 % thymol in 10 % sodium hydroxide solution.

Method.

To 2 cc. of blood, serum or plasma are added 6 cc. of water and 2 cc. of 20 % trichloroacetic acid solution, and the whole is well shaken. This mixture is then either centrifuged or filtered. Most brands of filter-paper contain traces of sulphate which vitiate the results. This sulphate is insoluble in water but soluble in dilute acid solution, so that before use all filter-papers should be washed first with dilute acid, then with distilled water and finally dried in an oven. Into a 15 cc. centrifuge tube 2.5 cc. of the filtrate are measured, followed by 5 cc. of 0.5 % benzidine in acetone, and well mixed. After standing half an hour the benzidine sulphate is centrifuged and the supernatant fluid removed. The precipitate of benzidine sulphate in the centrifuge tube is washed

twice with acetone followed by centrifuging. Finally the centrifuge tube is inverted over a sheet of filter-paper until it is dry. The benzidine sulphate in the centrifuge tube is dissolved in 1 cc. of *N* HCl, with heat if necessary. After cooling, 0.5 cc. of 0.1 % sodium nitrite is added followed one minute later by 2.5 cc. of 15 % sodium hydroxide. After thorough mixing, 2.5 cc. of the alkaline thymol solution are added and after standing 15 minutes the coloured solution is compared with a suitable standard prepared at the same time. The standard is prepared as follows. 2 cc. of one of the dilute standard solutions are measured into a test-tube followed by 1 cc. of nitrite solution. After standing 1 minute, 5 cc. of 15 % sodium hydroxide solution are added, the whole is well shaken, and then mixed with 5 cc. of alkaline thymol solution. For normal blood it is only necessary to put up the lowest standard (1 cc. $\equiv$ 0.0025 mg. S), but as a rule, to obtain standards approximating to the unknown, it is necessary to put up the whole series of standards.

As a preliminary, known amounts of ammonium sulphate were analysed in order to determine if the sulphate could be measured quantitatively by this method. The concentrations used were in the region of those sometimes found in plasma and serum, although higher than normal. These determinations were carried out under exactly the same conditions as in carrying out a determination with plasma and serum. From the results which are shown in Table I it will be seen that sulphate content could be quantitatively determined by this method with an error of less than 3 %.

Table I. *Determination of sulphate in standard solutions of ammonium sulphate.*

	mg. S per 100 cc. taken	mg. S per 100 cc. determined
1	0.91	0.90
2	1.83	1.79
3	0.91	0.93

Table II shows that added ammonium sulphate could be determined within an error of 5 %.

Table II.

	Inorganic sulphate content of blood-serum	Sulphate added	Total sulphate found	Sulphate recovered
1	1.55	1.14	2.67	1.12
2	1.55	2.28	3.95	2.40
3	1.55	4.56	6.22	4.67

The possible interference of phosphates and chlorides and of potassium oxalate, when plasma is used, must be taken into consideration. It was found, however, that 20 mg. P, 1 g. NaCl and 1 g. (COOK)₂ respectively per 100 cc. of blood, serum and plasma produced no appreciable effect on the determinations of sulphate.

In this work it was considered most convenient to express the sulphate content of blood in terms of S, to preserve the similarity with phosphorus and nitrogen.

The majority of workers have used serum or plasma in preference to whole blood for the determination of inorganic sulphate. Reed and Denis [1927], using a nephelometric method, state that in human blood the inorganic sulphate is found exclusively in the plasma. To test this point three different batches of normal oxalated blood were centrifuged and the cells washed twice with sulphate-free 0.85 % NaCl. 2 cc. of the corpuscular mass were mixed with 6 cc. of distilled water and then 2 cc. of 20 % trichloroacetic acid solution were added. After filtration the sulphate in the filtrate was estimated. No measurable coloration resulted. In order to make sure that the inorganic S had not dialysed through the red cell membrane the inorganic S contents of the whole blood and also of the plasma of nine different bloods were determined. Unfortunately haematocrit readings were not made but, averaging the values and assuming a cellular volume of 40 % of the whole blood, the inorganic content of the cells appears to be about one-fifth that of an equivalent volume of plasma.

Denis [1921] and Loeb and Benedict [1927] state that the retention of inorganic sulphate roughly parallels the retention of non-protein nitrogen, but Wakefield [1929, 2] states that there is no relation. It was decided to investigate this question. As a preliminary, the inorganic sulphate content of the sera of a number of normal healthy human subjects was determined. The results are shown in Table III. According to Wakefield [1929, 2] hospital patients suffering from diseases other than cardiac or renal are generally unsuitable for determining the normal range, since in many cases an increase in inorganic sulphate is observed even when there are no signs of renal inefficiency. It is extremely probable that in view of the extensive catabolic phenomena [Cuthbertson 1930, 1931], which result from injury, certain changes will occur in the blood, so that a great many hospital patients must therefore be excluded from any normal survey.

With four exceptions all the cases herein described were healthy men and women between the ages of 20 and 40 years.

Table III. *Sulphur content of normal plasma.*

	Case	Mg. S per 100 cc.	Case	Mg. S per 100 cc.	
Aet. 6 years	1	0.4	11	0.3	
13 "	2	0.4	12	0.1	
19 "	3	0.3	13	0.2	
	4	0.1	14	0.45	
	5	0.2	15	0.3	
	6	0.4	16	0.5	
	7	0.4	17	0.4	
	8	0.2	18	0.5	
	9	0.1	19	0.2	
	10	0.3	20	0.5	
			21	0.45	aet. 56

The inorganic sulphur content of the plasma or serum of normal fasting men and women appears to vary from 0.1 to 0.5 mg. per 100 cc. Other workers have obtained the following normal values expressed as mg. S per 100 cc.:

Denis [1921] plasma, 0.2–0.4 mg./100 cc.; Loeb and Benedict [1927] serum, 0.7–1.6 mg./100 cc.; Wakefield [1929] serum, 0.2–0.9 mg./100 cc.

The higher normal values quoted by Loeb and Benedict [1927] may perhaps be ascribed to the error of gravimetric estimation of amounts of barium sulphate of the order of 0.2 to 0.7 mg.

A few analyses of hospital cases are given in Table IV. They do not include any cases of obvious renal disease among their number.

Table IV.

Case	Disease	Mg. S per 100 cc.
22	Osteomalacia	0.5
23	Rheumatoid arthritis	0.2
24	Osteomalacia	0.9
25	Osteitis deformans	0.9
26	Fractured skull	0.7
27	Diabetes mellitus	0.9
28	Diabetes mellitus	0.7
29	Normal pregnancy	0.3

Forty cases of nephritis and cardio-renal disease have been investigated to determine what relation, if any, exists between the urea and inorganic sulphate content of the blood.

The urea content of the blood was determined on the whole fluid, but since this compound is contained in equal amount in plasma and red cells, the resultant values can be legitimately compared with the inorganic sulphate content of the plasma or serum.

Without considering particularly the various forms of nephritis and cardio-renal disease, but arranging the various cases in order of increasing retention of urea-nitrogen, it will be found that in general there is a coincident increase in the sulphur content and that on dividing the 40 cases into four arbitrary groups of 10 and determining the S : N ratios the values are fairly constant, 1 : 14.2, 1 : 14.7, 1 : 14.1 and 1 : 19.1. In the last group the sulphur increment lags behind the increment in urea-nitrogen (see Table V and Fig. 1).

Urea has been chosen for comparison with the inorganic sulphate, since its metabolism is in general parallel. It must be emphasised, however, that these are only average values and that the individual fluctuations are numerous and considerable. It is interesting to consider, however, that these ratios are of practically the same order as the S : N ratio of muscle, while those of the blood of normal cases are much lower (*ca.* 1 : 50).

If, however, there be deducted from these total retention values, the presumed normal content of urea-nitrogen and inorganic sulphate (15 mg. urea-N; 0.3 mg. inorganic S; S : N ratio 1 : 50), values are obtained which indicate that relative to urea-nitrogen, inorganic sulphate is retained in greater amount in the lower grades of retention, and that as the retention values increase, nitrogen relative to sulphur is retained in increasingly greater amount (S : N ratios 1 : 2.2, 1 : 8.1, 1 : 10.7 and 1 : 17.4).

From these data it appears that the measurement of the inorganic sulphate

content of the plasma or serum may prove to be of more service in the detection of mild degrees of renal impairment than the determination of the urea or non-protein nitrogen level.

Table V. *N mg. per 100 cc. whole blood; inorg. S mg. per 100 cc. plasma.*

Case	N.P.N.	Urea-N	Inorg. S	Case	N.P.N.	Urea-N	Inorg. S
30	32	13	0.3	40	58	22	1.8
31	30	15	0.6	41	52	23	2.6
32	35	15	0.8	42	40	23	0.6
33	56	15	2.3	43	63	23	1.2
34	41	16	1.6	44	63	27	1.6
35	33	17	1.0	45	70	28	0.9
36	43	18	1.8	46	80	29	4.0
37	39	20	0.4	47	60	32	1.4
38	41	20	0.9	48	59	33	2.9
39	52	22	2.3	49	80	36	2.3
Average		17	1.20			28	1.9
N : S		14.2 : 1				14.7 : 1	

Case	N.P.N.	Urea-N	Inorg. S	Case	N.P.N.	Urea-N	Inorg. S
50	56	37	2.9	60	109	60	5.1
51	87	39	3.1	61	150	70	6.0
52	69	40	3.4	62	115	83	6.1
53	64	42	2.4	63	106	88	4.2
54	79	48	4.8	64	137	100	4.2
55	71	50	2.9	65	132	111	4.9
56	82	50	2.4	66	174	120	5.7
57	112	50	2.4	67	228	132	6.3
58	100	50	2.4	68	209	158	8.9
59	109	55	5.2	69	236	177	6.1
Average		46	3.2			110	5.75
N : S		14.1 : 1				19.1 : 1	

Correlation coefficient between urea-N and inorg. S

$$M_{\text{urea-N}} = 50.25 \pm 4.309; M_{\text{inorg. S}} = 3.01 \pm 0.212$$

$$\sigma_{\text{urea-N}} = 40.405 \pm 3.047; \sigma_{\text{inorg. S}} = 1.993 \pm 0.1503$$

$$r_{\text{urea-N, inorg. S}} = +0.85 \pm 0.028$$

Fig. 1.

SUMMARY.

1. A method has been described for the determination of the inorganic sulphur content of the whole blood, plasma or serum. The method requires 2 cc. fluid.

2. Concentrations of phosphate, chloride and oxalate in the blood of the respective orders—20 mg. P, 1000 mg. NaCl and 1 g. $K_2C_2O_4$ per 100 cc.—do not affect the reaction.

3. There is very little inorganic sulphur in the cellular constituents of the blood.

4. The normal content for healthy adult men and women varies from 0.1 to 0.5 mg. inorganic S per 100 cc. plasma.

5. In cases of nephritis and cardio-renal disease there is on the average an increased retention of inorganic sulphur coincident with the increased retention of urea- and non-protein-nitrogen. Relative to nitrogen, inorganic sulphur is retained in proportionately greater amount in the minor degrees of retention than in the more severe types where relative to nitrogen it is less prominent.

The coefficient of correlation as will be seen from the calculation at the foot of Table V is $r_{\text{urea-N, inorg. S}} = + 0.85 \pm 0.028$.

In conclusion we wish to express out thanks to Dr G. M. Wishart for help with the statistical survey.

REFERENCES.

- Cuthbertson (1930). *Biochem. J.* **24**, 1244.
— (1931). *Biochem. J.* **25**, 236.
Denis (1921). *J. Biol. Chem.* **49**, 311.
Hubbard (1927). *J. Biol. Chem.* **74**, v.
— (1930). *J. Biol. Chem.* **88**, 663.
Kahn and Leiboff (1928). *J. Biol. Chem.* **80**, 623.
Loeb and Benedict (1927). *J. Clin. Invest.* **4**, 33.
Reed and Denis (1927). *J. Biol. Chem.* **73**, 623.
Wakefield (1929, 1). *J. Biol. Chem.* **81**, 712.
— (1929, 2). *Arch. Int. Med.* **44**, 244.
White (1923). *Amer. J. Physiol.* **65**, 537.
Yoshimatsu (1926). *Tohoku J. Exp. Med.* **7**, 553.