CCXXXI. DETERMINATION OF SMALL AMOUNTS OF SULPHUR AS SULPHATES IN BIOLOGICAL FLUIDS

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THREE main forms of sulphur are usually recognized in biological fluids: (a) inorganic sulphates, (b) ethereal sulphates and (c) neutral sulphur. The determination of these different forms of sulphur is usually made as sulphate. Several methods have been proposed but none of them is free from errors.

The earliest gravimetric methods depending on barium precipitation can only be used when large samples are available, as with urine. For blood sulphates barium precipitation has also been used, the precipitate being measured volumetrically [Hamburger, 1916; De Boer, 1917], or nephelometrically [Denis, 1921; Denis & Reed, 1926].

In other methods barium chromate is added and the liberated chromate estimated colorimetrically with diphenylcarbazide [Lang, 1929], or volumetrically [Klinke, 1924; Morgulis & Hemphill, 1932]. Øllgard [1934] estimated sulphates using an adsorption indicator (sodium rhodizonate).

An important advance was the use of benzidine proposed by Rosenheim & Drummond [1914] and applied by Raiziss & Dubin [1914] to the determination of sulphates in urine, and later to blood estimations by Fiske [1921]. Benzidine sulphate has been estimated by alkalimetric [Pohorecka-Lelesz, 1927; Cope, 1931; De Meio, 1933], iodimetric [Wakefield *et al.* 1931], manganimetric [Hoffman & Cardon, 1935] and colorimetric methods, the latter being based on conjugation reactions [Cuthbertson & Tompsett, 1931; Letonoff & Reinhold, 1936], or oxidation [Yoshimatsu, 1926; Hubbard, 1930; Wakefield, 1929].

All these methods are subject to error due to non-quantitative precipitation of benzidine sulphate and to the imperfection of the colour reactions employed. We therefore started the study of a new method, based on the use of benzidine as a precipitant but modifying the technique of precipitation so that complete removal of interfering substances was obtained. The benzidine sulphate is determined colorimetrically, using the weak reducing power of benzidine on phosphotungstomolybdic acid (Folin's phenol reagent). The Pulfrich photometer is employed for the final matching.

EXPERIMENTAL

Reagents.

Description of the method

Trichloroacetic acid: 10% aqueous solution.

Ammonia solution: prepared by diluting 20 ml. of ammonia (sp. gr. 0.91) to 100 ml. and adding 5 ml. of 0.02% methyl red.

Zirconium oxychloride or aluminium chloride ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$): 2.5% aqueous solution. We have used both reagents but we prefer the aluminium salt because it is more easy to obtain pure. Commercial zirconium oxychloride contains much sulphate which must be eliminated before use. It can be purified using Archibald's [1932] method: the zirconium oxychloride is dissolved in conc. HCl,

precipitated by making weakly alkaline with ammonia and boiled for a while. The precipitate is washed with dilute (1 %) HCl until free from sulphate. The washed precipitate is dissolved in hot conc. HCl and conveniently diluted.

The aluminium chloride used as a substitute for the zirconium salt can be obtained in a quite satisfactory degree of purity and used without previous purification.

Uranyl acetate: 0.4 % aqueous solution.

Benzidine: 1.5 % in anhydrous acetone. This solution is freshly prepared daily using pure benzidine and redistilled acetone.

Trichloroacetic acid: 5 % in anhydrous acetone. This solution should be freshly prepared since it does not keep more than a few days; after this time a yellow tinge appears.

Hydrochloric acid: 0.2 *N* and *N* (both approximate).

Gum arabic: 1 % solution. The gum arabic contains substances that reduce phosphotungstomolybdic acid, which can, however, be destroyed by oxidation with a few drops of bromine; before use, an adequate amount of the solution is boiled until completely free from bromine.

Phosphotungstomolybdic acid reagent: prepared according to Folin & Ciocalteu [1927].

Sodium carbonate: 16 % solution, prepared from the anhydrous salt of good quality.

Sodium sulphite: 2 % solution, must be prepared every day.

Benzidine hydrochloride: weigh 4.0068 g. of pure and dry benzidine hydrochloride, transfer to a 1000 ml. flask and dissolve in 0.2 *N* HCl to volume. Each ml. of this solution is equivalent to 0.5 mg. of sulphur. This solution keeps indefinitely.

As standard, a dilute solution is prepared by diluting 1 ml. of the concentrated solution to 50 ml. with 0.2 *N* HCl. Each ml. of this diluted standard corresponds to 0.010 mg. (10 μ g.) of sulphur. The diluted solution does not keep more than 2-3 days.

Glassware must be scrupulously clean and free from sulphates. As cleaning solution we use a mixture of 300 ml. of conc. HNO_3 with 1000 ml. of 10 % $\text{K}_2\text{Cr}_2\text{O}_7$.

Collection of blood and urine. The determination of sulphur may be carried out in whole blood, blood plasma or serum. For the determination in whole blood or blood plasma an anticoagulant must be used. As shown by Pohorecka-Lelesz [1927], potassium oxalate or sodium fluoride cannot be used since they precipitate with benzidine. Sodium citrate can only be used when the separation of the interfering substances with aluminium chloride or zirconium oxychloride is omitted. The best anticoagulant which can be used is heparin (see p. 1882, however). No special precautions are necessary for collecting the urine.

The sulphur of the blood

Inorganic sulphates. To 1 vol. of whole blood, serum or plasma add 2 vol. of 10 % trichloroacetic acid. Filter after 10-20 min. In a 15 ml. conical centrifuge tube measure 6 ml. of filtrate and add 0.5 ml. of 2.5 % aluminium chloride (or zirconium oxychloride) solution, thoroughly mix with a very thin glass rod and add 0.5 ml. of 20 % ammonia with methyl red. The solution must be distinctly alkaline. Let stand for a while, until aluminium hydroxide is flocculated. Centrifuge 5 min. at 2000 r.p.m. Any considerable excess of ammonia must be avoided, because some of the aluminium hydroxide may redissolve and be reprecipitated when the solution is acidified.

In another 15 ml. conical centrifuge tube, with a fine (2–3 mm.) end, put 3.5 ml. of the clear centrifuged solution and neutralize by adding 5 % trichloroacetic acid in acetone; about 1 ml. is necessary. Add 2 ml. more of the trichloroacetic acid in order to make the liquid distinctly acid and finally 6 ml. of 1.5 % benzidine solution in acetone.

Mix with the aid of a thin glass rod and keep in the ice box at 0° for 2 hr. or more. Centrifuge at about 3000 r.p.m. for 15 min.

The supernatant fluid is decanted by inverting the tubes and allowing them to drain for 5 min. on a filter paper moistened with acetone.

After this time, the precipitate is washed at the centrifuge with 10 ml. of acetone containing 5 % trichloroacetic acid, and the supernatant fluid is decanted and drained as before. The washed precipitate is dissolved in 2 ml. of 0.2 N HCl by heating in a water bath for a few minutes.

The solution is transferred quantitatively to a 25 ml. graduated tube with the aid of 3 or 4 washings with small quantities of water (1 or 2 ml.). The final volume must be about 6–8 ml.

The colour reaction is developed by adding the following reagents in the order specified and thoroughly shaking after each addition: add 0.5 ml. of gum arabic solution and 1 ml. of phosphotungstomolybdic acid reagent; let stand 3 min. or more, add 2.5 ml. of 16 % Na_2CO_3 and let stand at least 10 min.; finally add 2 ml. of recently prepared 2 % sodium sulphite; after a few minutes add water to make 20 ml.

The blue-grey colour produced is compared with a standard prepared simultaneously as follows. In two similar graduated test tubes put 1 ml. and 2 ml. of the standard benzidine solution equivalent to 10 and 20 $\mu\text{g.}$ of sulphur, add water to make 6 or 8 ml., followed by the reagents in the above-mentioned order.

The determination may be made with greater accuracy in a Pulfrich photometer using filter S. 72 (see p. 1888).

Total sulphates. In a small beaker put 6 ml. of blood filtrate and 0.5 ml. of N HCl; heat the beaker in a water bath until dry. Let cool and dissolve the residue in 6 ml. of distilled water, add 0.5 ml. of 2.5 % aluminium chloride and 0.5 ml. of 20 % ammonia. Centrifuge, take 3.5 ml. of the clear supernatant fluid, equivalent to 1 ml. of blood, and carry out the determination as indicated for inorganic sulphates.

The sulphur corresponding to the ethereal sulphates is calculated from the difference between that of the total and inorganic sulphates.

Total sulphur. In a 100 ml. Kjeldahl flask put 3 ml. of blood filtrate and add 3 ml. of pure conc. HNO_3 . Heat over gauze, with a Folin's fume absorber fitted to the flask, until the liquid turns brown and add a few drops of perhydrol. Continue the heating until a white ash is obtained. Let cool and add 6 ml. of distilled water, 2.5 ml. of aluminium chloride solution and 0.5 ml. of ammonia; centrifuge and complete the determination as for inorganic sulphates upon 3.5 ml. of the clear supernatant fluid, equivalent to 0.5 ml. of blood.

The neutral sulphur is calculated from the difference between the value obtained and that of the total sulphates-sulphur.

The sulphur of the urine

Preparation of the urine. The urine must be conveniently diluted and the interfering substances removed. Phosphates are the most important interfering substances in urine and can be removed according to Letonoff & Reinhold [1936] with uranyl acetate.

The greater the volume of urine excreted, the greater must be the quantity taken for the analysis. For urine of normal concentration, 1 ml. is transferred to a 50 ml. volumetric flask and 4 ml. of 0.4% uranyl acetate are added. The flask is thoroughly shaken and kept for 5 min. or more. At the end of this time the mixture is diluted to volume and filtered. This filtrate is used in all the determinations.

Inorganic sulphate. In a 15 ml. conical centrifuge tube put 1 ml. of filtrate equivalent to 0.02 ml. of urine and add 2 ml. of 10% trichloroacetic acid. The rest of the procedure is as indicated for blood.

Total sulphate. The determination is carried out on 0.5 ml. of filtrate equivalent to 0.01 ml. of urine; this is treated with 2-3 ml. of water and 0.5 ml. of *N* HCl and further treated as described for blood.

Total sulphur. 1 ml. of filtrate is incinerated with HNO_3 and perhydrol as described above; the ash is dissolved in 6 ml. of 10% trichloroacetic acid and 3 ml., equivalent to 0.01 ml. of urine, are taken for the analysis.

NOTES ON THE METHOD

Action of anticoagulants

The determination of sulphur in whole blood or blood plasma needs the use of an anticoagulant to prevent blood clotting. As pointed out above oxalates cannot be used and citrates can only be employed when the removal of interfering substances by aluminium chloride or zirconium oxychloride is omitted. Heparin is a good anticoagulant for inorganic sulphate determination, but is useless for ethereal and total sulphur determinations, because it contains sulphate which is set free during the hydrolysis or the destruction of the organic matter. Defibrination is quite satisfactory.

Deproteinization of the blood

Of the several deproteinizing reagents which have been proposed for sulphur determinations we have found trichloroacetic acid to be the most satisfactory when used in the manner specified.

When interfering substances are not removed, the quantity of trichloroacetic acid employed provides a distinctly acid filtrate (*pH* 2.8-3) of the most convenient acidity for the precipitation of sulphates with benzidine without simultaneous precipitation of benzidine phosphate.

When aluminium is used for removing interfering substances the filtrate is made alkaline and after separation of the aluminium hydroxide the liquid must therefore be reacidified with trichloroacetic acid-acetone before precipitating with the benzidine reagent.

Table I. *Comparison of the Letonoff-Reinhold and trichloroacetic acid techniques for deproteinization*

	Inorganic sulphates in mg. per 100 ml.	
	Trichloroacetic acid	Uranyl acetate
Human serum	1.45	1.54
	1.40	1.38
	1.60	1.56
Dog serum	4.97	4.91
	5.06	5.00

The deproteinizing technique with uranyl acetate, proposed by Letonoff & Reinhold [1936], offers no advantages over that used by us. Comparative results of both deproteinizing techniques upon the same blood sample are given in Table I. The results show little difference between the two methods. We have been unable to confirm the statement of Letonoff & Reinhold, that in trichloroacetic acid filtrates the inorganic sulphates increase on standing. We have analysed the same acid blood filtrate several days in succession without observing any alteration in the inorganic sulphate content.

Time of precipitation

The best conditions for complete precipitation of benzidine sulphate are a temperature of about 0° and a minimum time of about 1 hr.; longer times up to 24 hr. do not affect the results.

Washing of the precipitate

In contradiction to Hubbard's [1930] statement, we have found that neutral acetone dissolves an appreciable quantity of benzidine sulphate. Letonoff & Reinhold [1936], on the other hand, have found that the addition of 10 % of water to the acetone used in the washing produces a loss of sulphate-sulphur varying between 20 and 50 %.

We have found that acidifying the acetone used for washing with 5 % trichloroacetic acid gives satisfactory results, as can be seen from the comparative figures shown in Table II.

Table II. *Effect of washing the precipitate of benzidine sulphate*

Washing liquid	S as H ₂ SO ₄ μg.	S found μg.	Error %
Neutral acetone	10	9.30	- 7.0
	10	8.69	- 13.1
	10	7.54	- 24.6
Acetone plus 5 % trichloroacetic acid	10	9.95	- 0.5
	10	9.75	- 2.5
	10	10.00	0.0
	10	10.00	0.0

Removal of interfering substances

Several workers have recognized that the principal cause of error in sulphate determinations with benzidine is the presence of phosphates and chlorides.

The phosphates precipitate with benzidine as benzidine phosphate, particularly when the reaction of the solution is neutral or weakly acid. Fiske [1921] tried for the first time the separation of phosphates from sulphates with little success. When the quantity of phosphates is small their precipitation can be avoided by acidifying the solution to pH 2.5 or 3. In this way, sulphates of serum or plasma can be determined with a good degree of accuracy. Whole blood sulphates cannot be determined in the same way, because of the greater concentration of phosphates and other interfering substances.

For the removal of phosphates we first used the technique of Curtman *et al.* [1924] with zirconium oxychloride and later replaced it by the aluminium chloride method with similar results. The influence of phosphates upon sulphur determinations in pure mixtures of sulphates and phosphates with the benzidine reagent has been studied by us, with the results shown in Table III, where the effectiveness of the phosphate removal by zirconium oxychloride can be seen.

Table III. *Interference of phosphates in sulphate determination*

Mixture used		With phosphate removal by zirconium oxychloride		Without phosphate removal	
S as H_2SO_4 $\mu\text{g.}$	P as H_3PO_4 $\mu\text{g.}$	S found $\mu\text{g.}$	Error %	S found $\mu\text{g.}$	Error %
10	50	9.9	-1.0	12.90	+ 29.0
10	50	10.0	0.0	24.10	+141.0
10	100	9.9	-1.0	15.38	+ 54.0
10	100	10.0	0	12.50	+ 25.0

The results show that phosphates interfere with sulphur determination when their concentration is 5 times greater, or more, than that of sulphate. For smaller quantities of phosphates the interference is not appreciable because the trichloroacetic acid-acetone used in the washing removes practically all the benzidine phosphate.

The effectiveness of zirconium oxychloride and aluminium chloride in the removal of interfering substances is demonstrated in the experiments of Table IV

Table IV. *Recovery of sulphur added to dog's blood treated with zirconium oxychloride or aluminium chloride*

Precipitating agent	S in the blood sample $\mu\text{g.}$	S added $\mu\text{g.}$	S calculated $\mu\text{g.}$	S found $\mu\text{g.}$	Error %
Zirconium oxychloride	20.50	—	20.50	20.50	—
	20.50	10	30.50	31.44	+ 3.0
	20.50	20	40.50	39.80	- 2.2
Aluminium chloride	17.34	—	17.34	17.34	—
	17.34	10	27.34	27.05	- 1.1
	17.34	20	37.34	36.54	- 2.2

where known quantities of sulphur as sulphate were added to blood. Recovery of sulphates is quantitative with an error not greater than 3%. The equal efficiencies of zirconium oxychloride and aluminium chloride in removing the interfering substances were proved by treating two samples of the same blood filtrate with each of them and determining the sulphur (Table V).

Table V. *Efficiencies of zirconium and aluminium in removing interfering substances from blood plasma*

Mg. of inorganic sulphur per 100 ml.	
Zr treatment	Al treatment
3.43	3.41
3.43	3.37

Experiments carried out with blood without removing interfering substances give very high values for inorganic sulphate as shown in Table VI.

Table VI. *Determination of inorganic sulphur in blood, with and without the removal of interfering substances*

Dog's blood sample	Mg. of inorganic sulphur per 100 ml.		Increase produced by interfering substances %
	Without removing interfering substances	Removing interfering substances	
1	8.32	3.93	111
2	9.45	3.77	150
3	5.60	2.85	96

The acid-acetone used for washing removes practically all the benzidine phosphate of the precipitate so that some other unknown substances not removed by acid-acetone must be precipitated with the benzidine sulphate in the direct determination. This hypothesis is confirmed by the following facts: the results presented in Table III show that 100 μ g. of phosphorus as phosphate produce an error from +25 to +50 % in the sulphur determination, whilst in blood filtrates, with much less phosphate, the error is from +100 to +150 %.

In order to prove the existence of interfering substances other than phosphates, the following experiments were made.

Exp. 1. In a sample of blood, sulphates were precipitated without removing the interfering substances by the zirconium or aluminium treatment. The precipitate, washed once with neutral acetone, was dissolved by adding ammonia and heating. After cooling the benzidine was extracted with ether. The liquid, freed from ether by heating, was divided into two portions. One was analysed for phosphate by Fiske & Subbarow's method and with the strychnomolybdic reagent. No inorganic phosphorus could be detected. The other portion was ashed and the analysis for phosphorus gave positive results. This means that some organic phosphorus compound was precipitated along with the sulphates by the benzidine reagent, and retained by the precipitate without being removed by the neutral acetone used as washing fluid.

This experiment proves that the principal interfering substance or substances are organic compounds and that they contain phosphorus in combined form.

Exp. 2. A sample of blood was deproteinized with trichloroacetic acid and divided into three portions:

(a) One was precipitated directly and inorganic sulphates plus interfering substances determined. It gave 13.33 mg. S per 100 ml.

(b) The second fraction was precipitated with benzidine in the same manner. The precipitate was ashed and the residue dissolved and reprecipitated with benzidine. It gave 4.65 mg. S per 100 ml.

(c) The third fraction was treated with aluminium chloride to remove the interfering substances and sulphates precipitated with benzidine. It gave 4.56 mg. S per 100 ml. These results clearly demonstrate that the benzidine precipitate, ashed and reprecipitated, gave the same value as the direct determination after aluminium treatment.

The interfering substances are therefore destroyed during the ashing process and must be of organic nature.

These results speak for the great specificity of the benzidine as a sulphate precipitant when interfering substances had been previously removed.

The colour reaction

Some investigators who have used colorimetric determination of benzidine have employed oxidation reactions which are useless because of their uncontrollability. Others have employed azo derivatives of benzidine which are also useless because of their sparing solubility and lack of strict proportionality between colour and concentration of benzidine. Nobody, as far as we are aware, has hitherto employed reactions based on the weak reducing power of benzidine.

The colour produced between Folin's phenol reagent and benzidine is green at first and turns blue-green on standing for a long time. The reaction does not fade, it is proportional and suited for quantitative colorimetric analysis. As the reaction is carried out in alkaline solution a fraction of the phosphotungstomolybdic acid reagent is reduced by the benzidine sulphate, and the excess of reagent is completely destroyed by the alkali. This fact has permitted us to

bring the indefinite green colour of the reaction to a blue-grey stable colour by adding sodium sulphite after destruction of the excess of phenol reagent by alkali. This is the reason why 10 min. should elapse before adding the sodium sulphite which brings the colour to a definite maximum.

The colour obtained is blue-grey and not a pure blue, as in the direct reduction of phosphotungstomolybdic acid. The difference is due to the fact that oxidized benzidine forms a brown compound (probably a meriquinone), which remains in solution for some time, altering the blue colour of the reduced phenol reagent. As this compound precipitates on standing, we have been obliged to use as a protective colloid the gum arabic solution. Several previous trials demonstrated that 0.5 ml. of gum arabic solution, previously oxidized with bromine, is sufficient to prevent the precipitation of a quantity of benzidine 2 or 3 times larger than that present in the standard solution, and with the production of a blank reaction practically negligible for colorimetric determination.

When the photometric method is used, the quantity of gum arabic added may be greater if the blank used as compensating fluid is made with the same quantity of gum solution.

The action of sodium carbonate

The quantity of sodium carbonate to be added must be properly adjusted by previous titration of the phenol reagent. A deficit of sodium carbonate leaves excess of phenol reagent and the addition of sodium sulphite will reduce this excess to give more colour. An excess of sodium carbonate destroys the benzidine colour as may be seen in Table VII.

Table VII. *Influence of sodium carbonate*

Standard benzidine solution equivalent to S μg.	Sodium carbonate 16% ml.	Colorimetric reading mm.
10	2.0	13.2
10	2.2	17.2
10	2.3	19.9
10	2.4	20.1
10	2.5	20.0
10	2.6	20.0
10	2.7	20.1
10	2.8	20.0
10	3.0	21.1
10	3.3	22.0

For the titration of the phenol reagent the following procedure is used.

Quantities of standard benzidine solution equivalent to 10 μg. of sulphur are put in a series of graduated tubes; all the reagents are added as usual, except the sodium carbonate, which is added in increasing amounts from 2 to 3 ml. as is shown in Table VII. As can be seen from this table there is a zone of sodium carbonate concentration where the colorimetric reading is constant (in this case from 2.3 to 2.8 ml.); the mean value is taken as the optimal concentration of sodium carbonate to be added for 1 ml. of phenol reagent. 1 ml. of phenol reagent is usually enough and there is no advantage in using more. It suffices for more than 20 times the quantity of benzidine used as standard.

Influence of sodium sulphite

This reagent is employed to complete the reduction of that portion of the phenol reagent which is partly reduced by the benzidine. If the excess of phenol reagent has been destroyed with the proper quantity of sodium carbonate, excess

of sodium sulphite is not harmful. The usual quantity of sodium sulphite added, 2 ml. of a 2% solution, is more than enough for complete reduction of the reagent and the excess stabilizes the colour for long periods of time.

Proportionality of the colour reaction

The proportionality between the coloured reaction and sulphur concentration has been determined colorimetrically with the results expressed in Table VIII.

Table VIII. *Proportionality of the colour reaction*

Standard benzidine solution equivalent to S μg.	Theoretical reading mm.	Actual reading mm.		
		I	II	III
5	40.0	39.8	39.6	40.0
10 (test)	20.0	20.0	20.0	20.0
20	10.0	10.1	10.0	10.1
10	40.0	39.8	40.0	40.3
20 (test)	20.0	20.0	20.0	20.0
40	10.0	10.0	10.0	9.9

If the unknown is too dark to be compared with the standard, it can be diluted with water without change in the proportionality. Strict proportionality of the reaction has also been found in the photometric determination of the colour with the Pulfrich photometer (see p. 1888).

Accuracy of the method

The method was first checked by determination of a pure sulphuric acid solution, previously analysed by the barium sulphate method, and conveniently diluted for the assays. The results obtained are given in Table IX. Tables X and XI show the accuracy with which added sulphate may be recovered from blood and urine respectively.

Table IX. *Analysis of pure sulphuric acid solution*

S as H ₂ SO ₄ μg.	S found μg.	Error %	S as H ₂ SO ₄ μg.	S found μg.	Error %
5	5.00	0.0	10	10.05	0.5
5	4.95	-1.0	10	9.95	-0.5
5	5.00	0.0	15	14.70	-2.0
5	5.00	0.0	15	14.80	-1.3
8	7.87	-1.6	16	16.00	0.0
10	9.90	-1.0	20	19.60	-2.0
10	9.85	-1.5	20	20.00	0.0
10	10.00	0.0	20	19.80	-1.0

Table X. *Recovery of inorganic sulphates from dog's blood*

S in 1 ml. of serum μg.	S added μg.	S calculated μg.	S found μg.	Error %
14.38	5.0	19.38	19.23	-0.7
14.18	10.0	24.18	24.08	-0.4
17.39	5.0	22.39	21.96	-1.9
16.53	7.5	24.03	23.82	-0.9
16.53	22.5	39.03	37.50	-3.9
12.96	15.0	27.96	27.42	-1.9

Table XI. *Recovery of sulphates added to urine*

S in 1 ml. of urine μg.	S added μg.	S calculated μg.	S found μg.	Error %
555.5	250	805.5	770.0	-4.4
555.5	500	1055.5	1041.5	-1.3
570.5	250	824.5	819.5	-0.6

Photometric determination

The coloured solution does not possess a typical maximum absorption, but extinction increases steadily from the violet to the red portion of the spectrum; the most suitable filter is S. 72. As compensating liquid a blank is used with all the reagents in the same proportions as in the assay. The thickness of the layer used under ordinary conditions was 10 mm. but for concentrated solutions a smaller vessel can be used.

With the aid of the standard benzidine solution we have determined the calibration curve shown in Table XII.

Table XII. *Photometric calibration curve of S as benzidine equivalent*

Standard benzidine solution as S μg.	Extinction for 1 cm. layer	Specific extinction coefficient for 1 mg.
5	0.201	40.2
10	0.391	39.1
15	0.588	39.1
20	0.775	38.7
30	1.164	38.8
40	1.586	39.6
	Mean value	39.25

The photometric determination of sulphates presents advantages over the colorimetric method, not only because it is more accurate and requires no standard, but also because a larger amount of gum solution can be added to prevent the formation of the precipitate when high concentrations of sulphur are present.

The specific extinction coefficient for 1 mg. of sulphur was calculated from the analytical data obtained (Table XII) whose value is $E_{1\text{ cm.}} = 39.25$. Its constancy for different concentrations of benzidine equivalent to sulphur speaks for the strict proportionality of the reaction.

SUMMARY

A new micro-method for the estimation of the several known forms of sulphur in blood and urine is described. Quantities of sulphur of 5 μg. can be estimated with an error of 3 %.

Interfering substances are precipitated with aluminium, the sulphates with benzidine and the latter is estimated colorimetrically or photometrically by the reduction of phosphotungstomolybdic acid.

It is suggested that substances other than phosphates interfere in the usual methods of sulphur determination with benzidine.

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