XX. A VOLUMETRIC METHOD FOR THE ESTIMATION OF ETHEREAL AND INORGANIC SULPHATES IN URINE.

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(Received March 5th, 1914.)

Ever since the discovery by Baumann of the existence in urine of ethereal sulphates in addition to inorganic sulphates, their estimation has been carried out by weighing them as BaSO_4 on the principle of Baumann's original method, as modified in some more or less important details by subsequent investigators¹.

Until quite recently it has been assumed that the estimation of sulphuric acid as barium sulphate is one of the simplest and most trustworthy methods of analytical chemistry. The large amount of research which has been devoted by analytical chemists to this question [Hulett and Duschak, 1904; van 't Kruys, 1910; Allen and Johnston, 1910; Järvinen, 1913, etc.] has, however, abundantly proved that this assumption is erroneous. It has been conclusively shown that even in comparatively simple solutions the method is liable to yield inaccurate results. Without going into detail it may be stated that correct results can only be obtained by a careful adjustment of the conditions, and Allen and Johnston, indeed, attribute the many good results, which are obtainable by this method, to the happy neutralisation of inaccuracies.

If we consider that all these difficulties are met with when dealing with simple salt solutions, we are led to assume that they are still greater when we are dealing with sulphate estimations in such a complex organic fluid as urine. This was indeed recognised by O. Folin [1906] when he said: "The investigations described in this paper are the outcome of a conviction that the published records of sulphate and sulphur determinations in urine, including many of my own, are intolerably unreliable."

¹ The methods proposed by R. v. Lengyel (estimation as strontium sulphate) and by Freund (titration with barium acetate) do not seem to have acquired more than academical interest.

Folin has overcome by his careful work the difficulties inherent in the method, but there remains always the fact that, even at its best, the method is a very tedious one, requiring a great deal of time and experience if accurate results are desired.

We have worked out a volumetric method for the estimation of ethereal and inorganic sulphates in urine, which is based on the well-known insolubility of the sulphate of the base benzidine (*p*-diamino-diphenyl $\text{NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{C}_6\text{H}_4 \cdot \text{NH}_2$). The method seems to be free from the objections of the barium method and has the advantage of being very rapid, whilst the results are at least as trustworthy as those obtained by the Baumann-Folin method, which we have adopted as a standard.

The principle of the method depends on the fact that insoluble benzidine sulphate is precipitated from solutions of sulphates on the addition of a soluble benzidine salt. As benzidine is a weak base, its salts with strong acids are readily dissociated and the sulphuric acid contained in benzidine sulphate may be quantitatively titrated with standard alkali solutions, using phenolphthalein as an indicator. The method was introduced in this form into inorganic analysis by Raschig [1903] and its reliability was confirmed by v. Knorre [1905, 1910] and by Friedheim and Nydegger [1907]. Järvinen [1913] came to the conclusion that the benzidine method gives the most trustworthy results and is preferable to the old barium method in inorganic analysis.

Before the benzidine method can be applied to urine it is necessary to investigate if it fulfils the essential conditions which are demanded. Any reagent which is intended to replace barium chloride for the estimation of ethereal and inorganic sulphates in urine must fulfil three conditions. (1) It must precipitate in the cold only the inorganic sulphates, free from ethereal sulphates. (2) It must not precipitate any other urinary constituents. (3) The precipitation of the total sulphates after hydrolysis (= inorganic + ethereal sulphates) must be complete.

With regard to the first condition we have tested the behaviour of benzidine solutions towards several ethereal sulphates which we prepared synthetically according to Baumann [1878]. It was found that aqueous solutions of the potassium salts of phenylsulphuric acid, *p*-cresylsulphuric acid and resorcinolsulphuric acid behave towards benzidine hydrochloride exactly in the same way as towards barium chloride, i.e. the organically bound sulphuric acid is not precipitated in the cold, but readily after boiling for a short time with dilute hydrochloric acid. We have further tested the behaviour of the ethereal sulphates contained in normal urine towards

benzidine solutions in the following way. 200 cc. of urine were freed from inorganic sulphates by precipitation with barium chloride in the usual way. The filtrate which contained only the ethereal sulphates remained perfectly clear after the addition of a solution of benzidine hydrochloride. We must therefore conclude that under the ordinary conditions of urinary analysis the ethereal sulphates are not precipitated by benzidine.

With regard to the second condition we have found that the addition of a benzidine solution to normal urine results at once in the production of a bulky cream coloured precipitate, which on examination is seen to be partially crystalline and partially amorphous. It can easily be shown that this precipitate contains benzidine phosphate besides benzidine sulphate. This fact alone would seem *a priori* to condemn the method. (See also K. Spiro [Neubauer-Huppert, 1910], which is the only reference to the subject we came across in the literature.) We found, however, that urine which is faintly acidified with dilute hydrochloric acid, gives a crystalline precipitate which rapidly settles. This precipitate consists entirely of crystals of benzidine sulphate. It was tested qualitatively with negative results for phosphoric and uric acids, and the absence of other acids than sulphuric acid was proved by the close agreement of control analyses in which sulphuric acid determinations were carried out in the same sample by the barium method and by direct titration. The experiment described above, in which it was shown that the addition of benzidine solution to urine freed from inorganic sulphates produces no further precipitate, may also be quoted as evidence that sulphates are the only urinary constituents which are precipitated from acid urine by benzidine.

With regard to the completeness of the precipitation of sulphuric acid by benzidine in the presence of the other constituents of urine, we consider this proved by experiments in which added sulphuric acid was quantitatively recovered, and further by the fact that after the removal of the benzidine precipitate no further precipitate was produced by the addition of barium chloride.

Benzidine seems therefore to fulfil the conditions laid down above and we felt justified in proceeding to the actual quantitative estimations. It now became necessary to carry out a series of experiments in order to investigate (1) the influence of increasing quantities of hydrochloric acid on the accuracy of the result, (2) the minimum time necessary for complete precipitation, and (3) the quantity of benzidine solution required for complete precipitation.

The results of these experiments showed that excess of hydrochloric acid prevents the precipitation of benzidine sulphate and that a quantity sufficient

to produce an acid reaction to Congo red paper gives accurate results. Further it was found that the precipitation is complete after five minutes and that one hour's standing does not influence the result. Even a large excess of benzidine solution does not affect the accuracy of the result.

The ethereal and inorganic sulphates in urine may obviously be estimated in several ways by means of benzidine. (1) Inorganic and total sulphates may be estimated directly and the amount of ethereal sulphates be calculated by the difference between the two. (2) Total and ethereal sulphates may be estimated directly and inorganic sulphates calculated by difference. (3) Inorganic and ethereal sulphates may be estimated separately.

As the direct estimation of ethereal sulphates, owing to the small amount present, requires a relatively large volume of urine, which is not always available, we have estimated them indirectly by the difference between the total and inorganic sulphates, a procedure which is analogous to that employed by Folin in the barium chloride method.

In our experience the practical application of the benzidine method to urine does not meet with any difficulties if the precautions are observed, which have already been described by Raschig and by Järvinen in the case of inorganic analysis. They refer to care in filtration and washing of the precipitate. It is also important that an excess of free hydrochloric acid be avoided. Partly for this reason and partly in order to avoid the excessive formation of dark pigments which might interfere with the end point of titration, we have found it advisable to reduce the amount of hydrochloric acid in the hydrolysis of the ethereal sulphates to the minimum necessary for complete hydrolysis.

It is evident that the benzidine method can also be employed for the estimation of the neutral sulphur, by subtracting the quantity of total sulphates from that of the total sulphur. The latter may be estimated by applying the benzidine method to the final solution of mineral salts, as obtained by Wolf and Oesterberg's modification [1910] of Benedict's method [1909]. The presence of copper in this solution does not in any way interfere with the accuracy of the results.

By working with 0.01 N KOH solutions and methyl-red as an indicator it seems also possible to use much smaller quantities of urine and to convert the method into a micro-analytical method. Such a method would be of use in metabolic experiments on small animals and we are still engaged with experiments in this direction.

EXPERIMENTAL.

The preparation of the benzidine solution. 4 g. benzidine (Kahlbaum) are rubbed into a fine paste with about 10 cc. of water and transferred with about 500 cc. of water into a 2 litre flask. 5 cc. of concentrated HCl (spec. grav. 1.19) are added and the solution made up to 2 litres with distilled water. 150 cc. of this solution, which keeps indefinitely, are sufficient to precipitate 0.1 g. H_2SO_4 .

Influence of acidity, time and volume. The following series of experiments was carried out on a large sample of mixed urine. 25 cc. of urine were taken in each case. An estimation of inorganic sulphates by the barium method gave 0.104 % SO_3 .

Influence of increasing quantities of HCl (1 : 4).

HCl, cc.	Benzidine solution, cc.	Time of standing	0.1 N KOH, cc.	SO_3 %
1	100	10 mins.	6.45	0.103
2	"	"	6.50	0.104
3	"	"	6.30	0.101
4	"	"	6.00	0.096
5	"	"	5.60	0.089
7	"	"	4.60	0.074
10	"	"	2.20	0.035
15	"	"	none	—

This table shows clearly that an excess of hydrochloric acid prevents the formation of the benzidine sulphate precipitate. The quantity of hydrochloric acid necessary to produce an acid reaction with Congo red paper was 0.8 cc. In all the subsequent experiments we used therefore 1–2 cc. HCl (1 : 4).

Influence of time of standing.

HCl, cc.	Benzidine solution, cc.	Time of standing	0.1 N KOH, cc.	SO_3 %
2	100	5 mins.	6.40	0.102
"	"	10 "	6.45	0.103
"	"	15 "	6.40	0.102
"	"	20 "	6.60	0.107
"	"	30 "	6.45	0.103
"	"	45 "	6.45	0.103
"	"	60 "	6.50	0.104

These results prove that precipitation is complete after five minutes and that the result is not affected by one hour's standing. The short time

required for precipitation is a distinct advantage as against the lengthy period usually required before barium sulphate precipitates can be filtered.

Influence of volume of benzidine solution.

HCl, cc.	Benzidine solution, cc.	Time of standing	0.1 N KOH, cc.	SO ₃ %
2	70	5 mins.	6.50	0.104
"	100	"	6.40	0.102
"	150	"	6.50	0.104
"	180	"	6.50	0.104
"	200	"	6.50	0.104

The final result is evidently not influenced by an excessive quantity of the reagent and 100 cc. of the standard benzidine solution are sufficient to precipitate the whole of the sulphates contained in 25 cc. of normal urine.

An experiment may be mentioned here in which a known quantity of sulphate was purposely added to the urine. 2 cc. of a dilute sulphuric acid required 10.9 cc. 0.1 N KOH for neutralisation. This solution of sulphate, containing 53.4 mg. H₂SO₄, was added to 25 cc. urine which was previously found to contain 31.9 mg. H₂SO₄. In two experiments the final benzidine precipitates required 17.4 and 17.5 cc. 0.1 N KOH = 85.3 mg. and 85.4 mg. H₂SO₄.

	H ₂ SO ₄ added	H ₂ SO ₄ found
(1)	53.4 mg.	53.4 mg.
(2)	53.4 mg.	53.5 mg.

This experiment not only proves that the organic constituents of the urine do not interfere with the precipitation of sulphates by benzidine, but also that the precipitation is quantitative.

As an outcome of these preliminary experiments we have finally adopted the following procedure:

1. *The estimation of inorganic sulphates.*

25 cc. of urine are measured into a 250 cc. Erlenmeyer flask and acidified with dilute hydrochloric acid (1:4) until the reaction is distinctly acid to Congo red paper. Usually 1–2 cc. of dilute acid were added. 100 cc. of the benzidine solution (see above) are then run in and the precipitate, which forms in a few seconds, allowed to settle for ten minutes. The precipitate is filtered under pressure and washed with 10–20 cc. of water, saturated with benzidine sulphate. The precipitate and filter paper are transferred into the original precipitation flask with about 50 cc. water and titrated hot with

0.1 N KOH, after the addition of a few drops of a saturated alcoholic solution of phenolphthalein. 1 cc. 0.1 N KOH corresponds to 49 mg. H_2SO_4 .

2. *The estimation of total sulphates (inorganic and ethereal).*

The hydrolysis of the ethereal sulphates may be carried out in the usual way by gently boiling 25 cc. of urine with 20 cc. dilute HCl (1:4) for fifteen to twenty minutes. In this case it is necessary carefully to neutralise the solution after boiling and to add again dilute HCl until the reaction is acid to Congo red. We obtained, however, equally good results by reducing the quantity of acid used for hydrolysis to 2-2.5 cc. HCl (1:4), adding also 20 cc. of water to prevent undue concentration. The hydrolysis is complete after 15-20 minutes' gentle boiling and the precipitation of the cold solution may be proceeded with at once without previous neutralisation. It seems also that the smaller quantity of acid produces only very little pigment, which interferes to a slight extent with the end point, if the larger amount of acid is used.

The filtration and titration of the precipitate is carried out exactly as under 1.

In order to obtain accurate results, it is most important that the precipitate should be finely suspended in water before titration, and this again entails certain precautions during filtration, so as to prevent the caking together of the precipitate. We used a funnel of 6 cm. diameter and a perforated porcelain filter plate (5-7 mm.), covered either with paper pulp or with a well-fitting filter paper. Filtration and washing are carried out under pressure with the precaution of not allowing the precipitate at any time to be sucked dry on the filter. When the filtrate is no longer acid to Congo red and the precipitate together with the filter paper have been transferred to the original flask, it is necessary to shake it thoroughly before titration. Raschig recommended closing the flask with a rubber stopper for this purpose, a precaution which we found not essential. It is necessary to employ a stronger phenolphthalein solution than usual, as it is adsorbed to a considerable extent by the filter paper.

We have tested the accuracy of the method by comparing the results of the benzidine method with those obtained by Folin's gravimetric barium method. As many as seventy-five separate analyses were carried out. Most of the results agreed absolutely, the largest difference amounted to -0.0025% in favour of the barium method and to $+0.001\%$ in favour of the benzidine method (calculated as $\text{SO}_3\%$ of urine).

Some of the results, taken at random, may be quoted in full. 25 cc. of urine were used in each case.

	Gravimetric		Volumetric	
	Mg. BaSO ₄	% SO ₃	Cc. 0.1 N KOH	% SO ₃
(a) <i>Inorganic sulphates.</i>				
Urine 1	87.1	0.120	7.54	0.120
	86.7	0.119	(6 identical estimations)	
	Mean 0.1195			
Urine 2	57.6	0.079	4.90	0.078
	58.7	0.081	5.00	0.080
	0.080			0.079
Urine 3	85.7	0.117	7.28	0.116
			7.36	0.118
			7.32	0.117
			7.32	0.117
				0.117
(b) <i>Total sulphates.</i>				
Urine 3	93.1	0.128	7.99	0.128
			8.08	0.129
			8.08	0.129
				0.129

It will be seen that the results of the benzidine method agree not only *inter se* but also with those of the barium method as closely as can be expected¹.

SUMMARY.

A rapid and accurate method for the estimation of sulphates in urine consists in their precipitation with benzidine solution and the subsequent titration of the insoluble benzidine sulphate by means of 0.1 N potassium hydrate.

¹ A full account of this method was given at the November meeting of the Biochemical Society at King's College, London. Since this paper was ready for the press, our attention has been drawn to a publication on the same subject by R. Gauvin and V. Skarzynski in the December number of the *Bull. Soc. Chim.* p. 1121. It is satisfactory to note that these authors arrived at practically the identical method and results, although they do not seem to have systematically investigated the requisite conditions.

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