LVI. THE DETERMINATION OF SULPHATE.

A STUDY OF THE CONDITIONS NECESSARY FOR THE PRECIPITATION OF BENZIDINE SULPHATE, WITH SPECIAL REFERENCE TO THE ESTIMATION OF SULPHATES IN URINE.

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THE chief difficulties in the benzidine method for determination of sulphate arise in obtaining the conditions requisite for maximum precipitation of benzidine sulphate. Most investigators have drawn attention to the importance of adjusting the acidity of the solution, while some of them have studied the interference known to be caused by certain ions: chlorides, for example, may increase the solubility of benzidine sulphate [Fiske, 1921; Kahn and Lieboff, 1928].

Undoubtedly the phosphate ion has been the source of most error. The earlier workers, Raschig [1903; 1906], Friedheim and Nydegger [1907], Järvinen [1912], Gauvin and Skarzynski [1913], Rosenheim and Drummond [1914], Raiziss and Dubin [1914] and Drummond [1915], considered that approximate adjustment of the reaction of the solution to p_H 2 by addition of hydrochloric acid would eliminate precipitation of benzidine phosphate. However Fiske [1921], Yoshimatsu [1926], Hubbard [1927; 1930], Pohorecka-Leslesz [1927], Chatron [1931, 1, 2], Power and Wakefield [1931], Letonoff and Reinhold [1934], and Öllgaard [1934] found it advisable to remove phosphate. Quite recently however Kahn and Lieboff [1928] and Friedrich and Bauer [1934] reported that they found it unnecessary to remove phosphate. There is also some difference of opinion as to the best method of washing a benzidine sulphate precipitate.

The present work consists of an attempt to standardise the method of analysis, the chief points studied being:

(*a*) the influence of reaction on the precipitation of benzidine sulphate and phosphate;

(*b*) the influence of chlorides in affecting the solubility of benzidine sulphate;

(*c*) the influence of the concentration of benzidine hydrochloride;

(*d*) the influence of different methods of washing the precipitate.

EXPERIMENTAL.

The effect of hydrogen ion concentration on the precipitation of benzidine sulphate.

Benzidine hydrochloride was used to precipitate solutions of pure sodium sulphate (B.D.H. Analytical Reagent). Various hydrogen ion concentrations in the reaction mixture were attained by prior addition of either acetic acid-sodium acetate buffers or hydrochloric acid. The benzidine sulphate precipitated after 10 min. standing was titrated with 0·02N CO_2-free NaOH, using phenolphthalein as indicator. All pertinent quantitative analytical details are given in the tables of results. Filtrates from all determinations were collected and preserved in stoppered Erlenmeyer pyrex flasks. The p_H of each filtrate was determined using a Hildebrand hydrogen electrode, a saturated potassium chloride bridge and a Cambridge (Gallenkamp) potentiometer. Such apparatus makes possible the determination of p_H to within 0·1 unit.

Separation and washing of benzidine sulphate is a matter of some difficulty. Filtration by suction, unless very carefully controlled, may easily lead to a "caked" precipitate which dissolves only with difficulty in boiling alkali, thus causing great loss of time and uncertain end-points.

Filtering with suction on Jena 10 G 4 sintered glass filters is rapid and convenient, but requires so much attention as to become very tedious when many estimations are being done. Filtering on paper has the disadvantage that the paper has to be transferred to the titration flask with the precipitate; the disintegrated filter-paper absorbs the indicator and makes it difficult to judge the end-point.

Special filter-tubes were therefore devised made of 1 in. hard glass tubing drawn out at one end, to which 1 in. of $\frac{1}{8}$ in. glass tubing was fused, making a total length of 6 in.

Each tube was prepared for filtration as follows. A loosely fitting ball of glass-wool was placed in the tube and pushed into the narrow part by means of a brass wire 8 or 9 in. long and slightly narrower than the bore of the narrow tube. Strands of glass-wool projecting into the wider part of the tube were broken off by pressing the brass wire against the shoulder of the joint and rotating it. These strands were then shaken out of the tube.

Too little glass-wool will not grip the sides of the narrow tube, whilst too much will form a hard comminuted plug through which water will flow too slowly.

The plug was pushed along the narrow tube until it was about $\frac{1}{8}$ in. from the shoulder.

A properly made plug allowed water to flow through under gravity as a rapid succession of drops.

A suspension of ashless filter-paper in distilled water was poured into the filter-tube so as to fill $\frac{3}{4}$–1 in. above the plug. The tube was then filled with distilled water which was sucked through at the pump. Once all the water had passed through, a strong suction was applied in order to pack the paper-pulp tightly on top of the glass-wool. The rate of flow of the water was again tested. If the rate was about 2 drops per second the tube was ready for use.

The solution to be filtered was poured into the tube, the stream being directed against the side by means of a stirring rod. The tube, filled to within $\frac{1}{2}$ in. of the top, holds some 40 ml. For convenience in stirring the precipitate during subsequent washing, a stirring rod may be made with a hook about $1\frac{1}{2}$ in. from its upper end so that it may hang within the tube, without resting upon the filter-plug.

In the present determinations the filtrates were collected in 100 ml. Erlenmeyer flasks and tightly stoppered pending estimation of p_H.

As previous investigators have found, the precipitate could not be washed with water without considerable loss. Washing with 95 % acetone [Fiske, 1921] presented no advantages over washing, as recommended by Rosenheim and Drummond [1914], with a saturated solution of benzidine sulphate in water, 30–40 ml. of which, applied in two portions, were adequate.

After washing, the precipitate was transferred to a wide-mouthed flask by inverting the filter-tube so that its wide end was well inside the flask and dislodging the glass-wool plug by means of a rod. Both plug and precipitate were washed into the flask by means of a brisk jet of water from a wash-bottle. The tube was again inverted, scrubbed thoroughly with a rubber-tipped stirring rod and washed into the flask. Scrubbing and washing were repeated once.

The contents of the flask were then made up to 100 ml. with distilled water, phenolphthalein was added and the liquid titrated against 0·02 *N* NaOH as already indicated.

Blanks were subtracted from all determinations.

Results.

As will be seen from Tables I and II and Fig. 1 plotted from the data therein, variations in p_{H} between 1 and 5 exercise an effect upon the precipitation of benzidine sulphate.

Table I. *Effect of* p_H *on precipitation of benzidine sulphate.*

(Concentration of $SO_4^{=}$ before precipitation is the same as that of a normal human 24-hour urine specimen. Excess of benzidine is 100% in each case.)

No.	Vol. Na_2SO_4 sol. ml.	Vol. of buffer: *M* Na acetate ml.	Vol. of buffer: *M* acetic acid ml.	Vol. of water ml.	Vol. of benz. reagent 8 g./l. ml.	Sulphate: mg. S present	Sulphate: mg. S found	% error	p_{H} of filtrate
1	20	5·6	2·4	12	20	13·35	12·99	−3·1	4·7
2	20	5·6	2·4	12	20	13·35	13·02		4·7
3	20	3·2	4·8	12	20	13·35	13·04	−2·5	4·1
4	20	3·2	4·8	12	20	13·35	13·20		4·2
5	20	1·6	6·4	12	20	13·35	13·46	−0·5	3·6
6	20	1·6	6·4	12	20	13·35	13·28		3·6
7	20	0·2	7·8	12	20	13·35	13·36	0	2·15
8	20	0·2	7·8	12	20	13·35	13·33		2·15
9	20	—	—	20	20	13·35	13·28	0	2·0
10	20	—	—	20	20	13·35	13·36		2·0
		N HCl added, ml.							
11	20	4		16	20	13·35	13·22	−0·5	1·25
12	20	4		16	20	13·35	13·38		1·25
13	20	8		12	20	13·35	13·06	−2·0	0·94
14	20	8		12	20	13·35	13·12		0·96

In the experiments recorded in Table I the concentration of sulphate in the solution used was the same as that in a 24-hour specimen of normal human urine. It will be seen that the addition of 0·5 ml. of 2·5 *N* HCl to 5 ml. of the solution analysed (the minimum amount required to hydrolyse the ethereal sulphates of urine [Drummond, 1915]) is sufficient to reduce the p_{H} of the reaction mixture to 1 and to cause an error of −2% (approx.).

In more dilute solutions the effect of p_{H} on percentage recovery of sulphate is more marked (Table II).

This is further shown in the experiments in Table III. Here the p_{H} and the amount of benzidine hydrochloride used are constant, and the percentage of $SO_4^{=}$ precipitated diminishes as the concentration of the sulphate becomes less.

Table II. *Effect of* p_H *on precipitation of benzidine sulphate from dilute sulphate solution.*

(Concentration of $SO_4^{=}$ before precipitation was one-quarter of that of a 24-hour specimen of normal human urine. Excess benzidine 108% in each experiment.)

		Vol. of buffer				Sulphate			
No.	Vol. of Na_2SO_4 sol. ml.	*M* Na acetate ml.	*M* acetic acid ml.	Vol. of water ml.	Vol. of benz. reagent 8 g./l. ml.	mg. S present	mg. S found	% error	p_H of filtrate
1	20	5·6	2·4	27	5	3·358	1·78	- 47·0	4·8
2	20	5·6	2·4	27	5	3·358	1·82	- 46·0	4·8
3	20	3·2	4·8	27	5	3·358	2·53	- 24·7	4·3
4	20	3·2	4·8	27	5	3·358	2·54	- 24·4	4·2
5	20	1·6	6·4	27	5	3·358	3·08	- 8·4	3·8
6	20	1·6	6·4	27	5	3·358	3·07	- 8·5	3·8
7	20	0·8	7·2	27	5	3·358	3·24	- 3·5	3·3
8	20	0·8	7·2	27	5	3·358	3·27	- 3·0	3·3
9	20	0·2	7·8	27	5	3·358	3·30	- 2·1	2·55
10	20	0·2	7·8	27	5	3·358	3·29	- 2·1	2·55
		N HCl added, ml.							
11	20	None		35	5	3·358	3·28	- 2·5	2·3
12	20	None		35	5	3·358	3·28	- 2·5	2·3
13	20	2		33	5	3·358	3·02	- 10·2	1·34
14	20	2		33	5	3·358	3·00	- 10·8	1·34
15	20	4		31	5	3·358	2·62	- 22·0	1·1
16	20	4		31	5	3·358	2·65	- 21·3	1·1
17	20	8		27	5	3·358	1·92	- 43·3	0·85
18	20	8		27	5	3·358	1·94	- 42·8	0·85
19	20	8		27	5	3·358	1·92	- 43·3	0·84
20	20	35		0	5	3·358	0	-100	0·21
21	20	35		0	5	3·358	0	-100	0·21

Table III. *Effect of concentration of sulphate on benzidine sulphate precipitation.*

(Concentration of benzidine, before precipitation, is kept constant, as also is p_H.)

			Sulphate				
No.	Vol. of Na_2SO_4 sol. analysed ml.	Vol. of benz. reagent ml.	mg. S/ 100 ml. present	mg. S/ 100 ml. found	% error	mg. S/l. remaining in sol.	p_H of filtrate
1	40	10	166·9	165·6	- 0·8	1·4	2·14
2	40	10	166·9	164·5	- 1·5	2·6	2·13
3	40	10	84·0	84·6	+ 0·7	—	2·15
4	40	10	84·0	83·3	- 0·8	0·7	2·13
5	40	10	41·8	39·9	- 4·5	1·9	2·15
6	40	10	41·8	40·1	- 3·9	1·6	2·20
7	40	10	41·8	39·8	- 4·8	2·0	2·18
8	40	10	21·0	18·3	-12·9	2·7	2·05
9	40	10	21·0	19·0	- 9·6	2·0	2·05
10	140	35	8·4	6·95	-16·8	1·4	2·26
11	140	35	8·4	7·13	-14·6	1·3	2·27

Effect of excess of benzidine hydrochloride.

It should be emphasised here that the final concentration of benzidine hydrochloride remaining in excess is important independently of p_H in affecting the completeness of precipitation. Thus the shape of each curve in Fig. 1 is determined by p_H, but the position of curve *B* in relation to curve *A* is due to the fact that the excess of benzidine in the case of curve *A* is four times as great as in the case of curve *B*.

For the purposes of sulphate estimation a strong benzidine hydrochloride reagent which provides a large concentration of unprecipitated benzidine ions is favourable. A practical limit is however set to the strength of the benzidine reagent by the fact that strong reagents favour co-precipitation of benzidine hydrochloride and therefore lead to high results.

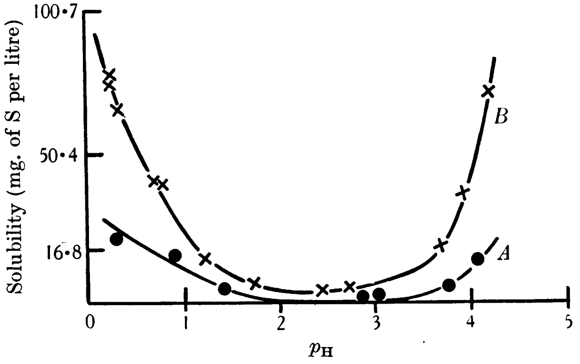

Fig. 1. Variation of solubility of benzidine sulphate with varying p_H.

Benzidine acetate was tried as a precipitating reagent but was unsatisfactory owing to the difficulty of attaining the optimum p_H save in presence of a large excess of acetic acid; substitution of chloroacetic acid as acidifying agent was of no assistance.

The effect of p_H *on the precipitation of benzidine phosphate.*

Solutions of KH_2PO_4 (Analytical Reagent, B.D.H.) were prepared. Rosenheim and Drummond's [1914] benzidine reagent (containing 2 g. base per litre and having p_H 1·94) was added. The mixture was kept for 10 min. after which the precipitated benzidine phosphate was filtered off on paper or on sintered glass filters. The p_H of the filtrate was determined electrometrically. The precipitate was titrated at the boiling-point with standard NaOH using phenolphthalein as indicator; calculations of the phosphate precipitated were made on the assumption that red coloration of the indicator represented approximately complete conversion of H_3PO_4 into Na_2HPO_4.

The concentration of the phosphate solutions used varied between 1·3 and 0·45 mg. P/ml.; 1 mg./ml. is the concentration of P normally present in a 24-hour sample of human urine [Hawk (Bergeim), 1931]. Variations of p_H were obtained by prior addition of either acetate buffers or hydrochloric acid.

The results in Table IV show that some benzidine phosphate may be precipitated even when the p_H is as low as 1·4 (no. 12 of Table IV). Slight variations in p_H exercise a marked effect on the precipitation of benzidine phosphate, the percentage precipitated increasing rapidly as the p_H changes from 2 to 4. At p_H 2·75, which is optimum for the precipitation of benzidine sulphate, considerable amounts of benzidine phosphate are precipitated.

Table V shows the extent to which the presence of phosphate may interfere in the estimation of sulphate by the benzidine method. In these experiments a solution containing 2·961 g. Na_2SO_4 and 3·993 g. KH_2PO_4 was used; the concentrations of $SO_4^=$ and of $PO_4^{\equiv}$ were thus about the same as in normal human 24-hour urine. To this solution benzidine hydrochloride reagent was added.

Table IV. *Effect of* p_H *on precipitation of benzidine phosphate from pure phosphate solutions.*

(Normal human 24-hour urine contains 1 mg. P per ml.)

No.	Vol. of KH_2PO_4 sol. ml.	Vol. of benz. reagent 2 g./l.	Phosphate mg. P Present	Phosphate mg. P Found	% precipitated	p_H of filtrate
1	20	80	9·03	None	0	1·4
2	20	80	9·03	0·7	7·7	2·3
3	20	80	9·03	None	0	2·35
4	20	80	9·03	3·8	42	2·4
5	20	80	9·03	5·2	58	2·6
6	20	80	9·03	4·7	52	2·6
7	20	80	9·03	5·9	66	3·6
8	20	80	9·03	6·6	73	4·2
9	20	80	25·08	0·40	1·6	1·8
10	20	80	25·08	12·1	48·2	2·0
11	150	60	12·54	6·98	55·7	3·2
12	5	20	4·5	0·37	8·2	1·4
13	5	20	4·5	1·18	26·2	2·2
14	5	20	4·5	2·12	47·1	2·3
15	5	20	4·5	2·23	49·6	2·4

Table V. *Effect of the presence of phosphate on the estimation of sulphate.*

No.	Vol. of Na_2SO_4 + KH_2PO_4 sol. ml.	Vol. of water added ml.	Vol. of 2·5*N* HCl added ml.	Vol. of benz. reagent added 8 g./l.	Sulphate mg. S Present	Sulphate mg. S Found	% error	Phosphate present in precipitate mg. P	p_H of filtrate
1	20	20	None	20	13·36	14·09	+5·4	0·48	1·9
2	20	20	,,	20	13·36	14·09	+5·4	0·34	1·9
3	20	20	,,	20	13·36	14·0	+4·5	0·40	1·9
4	20	20	,,	20	13·36	13·96	+4·2	0·36	1·9
5	20	20	,,	20	13·36	14·09	+5·4	0·48	1·9
6	20	20	,,	20	13·36	14·20	+6·0	Not analysed	1·9
7	20	20	,,	20	13·36	13·89	+3·7		1·9
8	20	18·4	1·6	20	13·36	13·11	−1·9		1·1
9	20	18·4	1·6	20	13·36	12·96	−3·0		1·1

The precipitate was filtered, washed and titrated in the usual way, the p_H of the filtrate being determined electrometrically.

In nos. 1–5 (Table V) the amount of benzidine phosphate precipitated was estimated quantitatively by the method of Tisdall [1922] after solution of the precipitate in excess of alkali and removal of the benzidine by extraction with ether.

As will be seen from Table V at p_H 1·9 enough benzidine phosphate was precipitated to cause an error of +4 % to +6 % in the sulphate estimation. In nos. 8 and 9, where an amount of HCl was added sufficient to reduce the p_H to 1·1, the sulphate recovery was 2–3 % low owing to incomplete precipitation of benzidine sulphate.

Even at a p_H as low as 1·1 the precipitated benzidine sulphate was not free from contamination with benzidine phosphate as was shown by quantitative analysis of precipitates obtained at this p_H for phosphate.

In addition to p_H, time of standing before filtration and variations in temperature exercise marked effects on the precipitation of benzidine phosphate. Precipitation of benzidine sulphate is complete in 10 min. and is not affected by a longer time of standing (see also Rosenheim and Drummond [1914]), but the filtrates from a mixed benzidine phosphate-sulphate precipitate often deposited benzidine phosphate on standing. The error in sulphate estimations due to contamination with benzidine phosphate was greatly increased if the reaction mixture was kept 24–48 hours before filtration. Variations in laboratory temperature were also found to affect the amount of benzidine phosphate precipitated. At lower temperatures larger amounts were precipitated.

Removal of phosphate.

Three different methods of phosphate removal were investigated, *viz.* those of Fiske [1921], Yoshimatsu [1926] and Hoffmann and Cardon [1935].

Hoffman and Cardon's method, originally designed for serum, in which the coagulation of a ferric hydroxide solution is made use of, was useless since the coagulum could not be washed free from sulphate [*cf.* Freundlich, 1928].

Both Fiske's method, in which phosphate is removed as $MgNH_4PO_4$, and Yoshimatsu's method, in which phosphate is removed by means of uranyl acetate in the presence of acetate buffer at p_H 5, gave precipitates which were readily washed free of sulphate and filtrates which were free of phosphates when tested with strychnine molybdate. Using a dilution of 1 : 5 in removing phosphate and employing the benzidine hydrochloride reagent of Fiske, both the latter's and Yoshimatsu's method were found to give results concordant with those obtained by the method of Folin [1905], when applied to a solution of Na_2SO_4 and KH_2PO_4 (B.D.H. Analytical Reagents). These two methods were both tested on a 24-hour sample of human urine. The results are shown in Table VI. The p_H in these experiments was attained by making the solution, before addition of the benzidine reagent, just yellow to bromophenol blue by means of drops of N HCl.

Table VI. *Analysis of human urine (24-hour sample) by the benzidine method after removal of phosphate.*

Method of removal of phosphate	Vol. of original urine analysed ml.	Vol. after phosphate removal ml.	Titration 0·0172 N NaOH ml.	S found mg./l.	p_H of filtrate
$MgNH_4PO_4$	4	20	13·56 13·62 13·55	929 932 927	2·7 2·5 2·5
Uranyl acetate	4	20	13·53 13·66 13·66	927 936 936	2·8 2·9 3·1
		Wt. of $BaSO_4$ (g.)			
Phosphate not removed. Folin's gravimetric method	30	0·2012 0·2014	—	921·5	—

The method of Fiske, since it does not involve heating the urine (which is essential to the uranium method), seems to be preferable.

Effect of chlorides.

Fiske [1921] and Kahn and Lieboff [1928] state that the presence of NaCl increases the solubility of benzidine sulphate. Fiske showed that with a solution, of which the sulphate concentration was comparable with that of normal urine, analysis by Drummond's method might lead to 97 % precipitation of sulphate if the Cl : S ratio was 30 : 1 or to 91 % precipitation if the ratio were 60 : 1. Kahn and Lieboff report an error of −25 % when the Cl : S ratio is 90 : 1.

The Cl : S ratio in a 24-hour sample of normal human urine is 7 : 1 [Hawk (Bergeim), 1931]. Experiments were therefore performed in which sulphates were precipitated by benzidine hydrochloride from Na_2SO_4 solutions containing varying amounts of NaCl. The results are given in Table VII from which it will be seen that, when Cl^- and $SO_4^=$ are present in the concentrations found in normal human 24-hour urine, the results obtained are accurate within the limits of experimental error. When the sulphate solution is more dilute, however, chloride even in the amount present in normal urine prevents complete precipitation of benzidine sulphate.

Table VII. *Effect of chloride on precipitation of benzidine sulphate.*

No.	Vol. of Na_2SO_4 sol. analysed ml.	S present in experiment mg. S	Chloride present mg. Cl	Cl:S ratio	Vol. of benz. reagent 8 g./l. ml.	S found mg.
1	20	6·67	0	0	10	6·66
2	20	6·67	0	0	10	6·68
3	20	6·67	243	36·4	10	6·66
4	20	6·67	243	36·4	10	6·65
5	20	6·67	61	9·1	10	6·63
6	20	6·67	61	9·1	10	6·66
7	40	3·36	0	0	5	3·22
8	40	3·36	0	0	5	3·22
9	40	3·36	122	36·3	5	3·12
10	40	3·36	122	36·3	5	3·06
11	40	3·36	486	145	5	2·88
12	40	3·36	486	145	5	2·82

SUMMARY.

1. In an aqueous medium benzidine sulphate has minimum solubility at p_H 2·75 ± 0·3. In analysis it is therefore desirable to attain this p_H in the reaction mixture.

2. Benzidine phosphate is precipitated from solutions of potassium phosphate similar in concentration to a normal human 24-hour urine sample at p_H values as low as 1·4 and the amount of phosphate precipitated increases rapidly with p_H. In analysis of urine it is therefore desirable to remove phosphate. Either the method of Fiske [1921] or that of Yoshimatsu [1926] is suitable for this purpose.

3. The excess of benzidine left unprecipitated has an important effect in decreasing the solubility of benzidine sulphate. The supernatant fluid should contain about 1 g. benzidine per litre.

4. In sulphate solutions of concentration comparable with a normal human 24-hour urine sample, chloride in amounts much larger than physiologically normal does not interfere. In more dilute sulphate solutions chloride leads to low results.

5. A saturated solution of benzidine sulphate in water is a convenient and suitable liquid for washing the precipitated benzidine sulphate.

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