XCIH. THE ESTIMATION OF ACETOACETIC ACID AND β -HYDROXYBUTYRIC ACID IN URINE.

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THE principal methods for estimating acetoacetic acid, acetone and β -hydroxybutyric acid in body fluids are those of Shaffer-Marriott [1914] and of Van Slyke [1917].

The latter is a gravimetric estimation depending on the formation of a complex mercury-acetone compound, $3\text{HgSO}_4 \cdot 5\text{HgO} \cdot 2(\text{CH}_3)_2\text{CO}$. The former is an iodometric method based on the Messinger-Huppert titration.

In both methods the acetoacetic acid is decomposed with sulphuric acid, thus

and the β -hydroxybutyric acid is oxidised by a mixture of $\text{K}_2\text{Cr}_2\text{O}_7$ and H_2SO_4 , thus

In the Shaffer-Marriott method the acetone is made to react with hypiodite to form iodoform; in the Van Slyke method it combines with mercuric sulphate to form the complex mercury-acetone compound.

For the estimation in urine the precipitation of pigments and sugar and any other aldehyde-forming substances is a necessary preliminary. Shaffer-Marriott used the lead acetate-ammonia method; Van Slyke used the copper sulphate-calcium hydroxide method.

This preliminary precipitation in urine is of the greatest importance in the estimation of β -hydroxybutyric acid, but is not absolutely essential in that of acetoacetic acid and acetone by the Shaffer-Marriott method. This statement is based on a large number of comparative estimations. In the Van Slyke method precipitation must always be carried out.

Hence in purely clinical work, using the former method, where only the acetoacetic acid and acetone are estimated, immediate distillation in acid solution with subsequent iodometric titration is all that is necessary. The yield in both methods for acetoacetic acid and acetone is generally agreed upon to be between 90 and 95 %.

In the estimation of β -hydroxybutyric acid, however, in which oxidising agents are used, preliminary precipitation can never be dispensed with, as the interfering substances present are fatal to accurate results. Even after

precipitation it is by no means certain that what we are estimating is only this hydroxy-acid, although it is certainly the main constituent in, for example, urines of advanced diabetics.

In order to remove aldehydes from the distillate obtained from the oxidation, Shaffer-Marriott re-distilled with alkaline hydrogen peroxide before titration, a method which gives very constant yields of about 85 %. This percentage is not attained when dealing with quantities in the region of 5 mg. %. In the Van Slyke method the β -hydroxybutyric acid is obtained by difference, dichromate being used with one sample and not with a second, the difference in mass of the two precipitates representing the hydroxy-acid.

The principal objection to both these methods, particularly if serial determinations are to be made, is the long time necessary for a single analysis.

The Shaffer-Marriott method cannot be done under 3 or 4 hours and Van Slyke's method cannot be finished under $2\frac{1}{2}$ to 3 hours. The high resistance of β -hydroxybutyric acid to oxidation is the main cause of these protracted periods.

In Van Slyke's method the filtration through the Gooch crucible is somewhat tedious and the transference of small particles of precipitate is always troublesome. In spite of this, however, we consider the Van Slyke a much more tractable method than the Shaffer-Marriott.

A further point about these methods is the relatively large volumes of urine used. In the Shaffer-Marriott an average of 50 cc. is taken and in the Van Slyke it is best to use 25 cc.

If, as in the work for which these methods were studied, only small volumes are available on which several analyses have to be made, such methods are not satisfactory.

Lastly, we may refer to the considerable expenditure of chemicals incident to these methods.

Engfeldt, using a micro-method and very small quantities of β -hydroxybutyric acid, has obtained yields of 70 % from urine and blood. Lublin [1922], combining the methods of Shaffer-Marriott and Engfeldt, used fractional volumes of urine and blood and claimed a fair accuracy for his micro-method.

The low yield in hydroxy-acid is due to the fact that the oxidation involves not only a production of acetoacetic acid and acetone but also a decomposition of acetoacetic acid with the production of acetic acid, in accordance with the well-known acid hydrolysis of ketonic acids. Thus:

Van Slyke has shown that, when $\text{K}_2\text{Cr}_2\text{O}_7$ and H_2SO_4 act upon β -hydroxybutyric acid at room temperature for some time, practically only acetic acid is produced; but at boiling temperature acetone is produced in considerable quantities.

In Lublin's method no preliminary precipitation of pigments is carried out in the analysis of urine, but the Folin-Wu tungstic acid precipitation is

applied to the estimation in blood. For β -hydroxybutyric acid he assumes Engfeldt's yield of 70 %. He states that precipitation is unnecessary for ordinary purposes. This is, in our experience of this method, true for the estimation of the acetoacetic acid and acetone, but certainly not so for that of β -hydroxybutyric acid.

The volumes used by Lublin were 0.5 cc. urine and 0.2 cc. blood. In cases of severe acetonuria there is certainly no difficulty with 0.5 cc. urine, but 0.2 cc. blood has, in our hands, not yielded results which could be relied upon except in cases of severe diabetic ketosis.

Lublin distils directly into alkaline hypiodite. Using such small volumes of material it would be futile to attempt re-distillation with alkaline H_2O_2 as in the Shaffer-Marriott method and so it is dispensed with. With regard to time of distillation Lublin gives 10 minutes for each stage, *i.e.* 10 minutes' distillation with acetic acid for the acetoacetic-acetone fraction and then a further 10 minutes' oxidation and distillation with $\text{K}_2\text{Cr}_2\text{O}_7$ and H_2SO_4 .

Our experience of this method has shown that perceptible differences from the blank of the apparatus and reagents can be detected up to as long as 30 minutes from the commencement of each distillation, provided that rather larger volumes of distilled water are used in a larger distilling flask. Twenty minutes has been found the minimum time for a maximum yield.

In the oxidation for the β -hydroxybutyric acid, Engfeldt obtained his yield of 70 % using 1 g. of $\text{K}_2\text{Cr}_2\text{O}_7$ in 2 % solution at constant volume of urine.

Lublin used 20 cc. of 2 % $\text{K}_2\text{Cr}_2\text{O}_7$ in 20 % H_2SO_4 .

The strength of dichromate used is of the first importance and we have found, using various solutions, that that used by Lublin is satisfactory. Of course the yields become less as we decrease the concentration of the β -hydroxybutyric acid, but for a given solution fairly constant results are obtainable.

Variations in time of oxidation and in strength of flame give rise to considerable variations in yield.

The method which we used in certain investigations to be published later was a modification of that proposed by Lublin.

The apparatus is a simple one and consists of a 250 cc. Kjeldahl flask, provided with an inlet tube made by fusing a long piece of thin glass tubing to a drawn-out test tube; the glass tubing is drawn to capillary bore and cut so as to reach almost to the bottom of the flask. Through this an air current can be drawn through the contents of the flask, and the dichromate-sulphuric acid mixture can also be introduced.

The flask is connected by a long delivery tube to a condenser which is connected to a 250 cc. Erlenmeyer flask, fitted with a glass tube with a perforated bulb and with a dilated tube, the latter being connected to a suction pump by a rubber tube. The rubber tube carries a screw clip for regulation of the air current. The Bunsen flame used for heating the flask is surrounded by a guard and can be adjusted by means of a screw clip.

Solutions required.

1. Basic lead acetate solution prepared according to Shaffer's directions; 180 g. lead acetate are dissolved in about 500 cc. distilled water and boiled; 110 g. of litharge are then gradually added until a clear solution is obtained with no remnants of the oxide. This is made up to a litre with distilled water. This solution on standing deposits some of the basic salt but the supernatant fluid is clear. The pipette used for this solution should be dry and should be cleaned immediately after use.

2. Strong solution of ammonium hydroxide (Sp. 0.880).
3. Solution of 25 % H_2SO_4 .
4. Solution containing 2 g. $\text{K}_2\text{Cr}_2\text{O}_7$, 20 cc. concentrated H_2SO_4 , made up with distilled water to 100 cc.
5. $N/50$ iodine
6. $N/200$ $\text{Na}_2\text{S}_2\text{O}_3$ freshly prepared from $N/10$ thiosulphate.
7. 25 % solution of nitrite-free NaOH .
8. 20 % HCl containing no free chlorine.

Into a small beaker 4 cc. urine¹, 4 cc. distilled water and 4 cc. of basic lead acetate solution are put and after a little agitation 2 cc. of ammonia are run in from a burette. Sugar, pigments, etc. are thus precipitated. In the case of urines containing little sugar less acetate is added and more distilled water, but the final volume is always 14 cc. After standing for 5 minutes the mixture is filtered into a test tube when a clear, colourless filtrate is obtained. 40 cc. distilled water are placed in the Kjeldahl flask and 3.5 cc. filtrate are run in from an accurately graduated pipette. This volume of filtrate is equivalent to 1 cc. urine. The volume of filtrate obtained depends on the amount of sugars, etc. to be precipitated. If this is inconsiderable as much as 8 cc. filtrate are obtained, but with urines of high sugar content a bulky precipitate is produced and perhaps as little as 4 or 5 cc. are obtained. In general, no difficulty is found in obtaining 3.5 cc. for the estimation.

5 cc. of 25 % H_2SO_4 are now added. If an excess of lead acetate is present a white precipitate of PbSO_4 is thrown down, but with a little practice this can be avoided by adjusting the volume of lead acetate added.

In the Erlenmeyer flask are placed 40 cc. distilled water, 10 cc. $N/50$ iodine and 2 cc. 25 % NaOH .

A slow current of air is drawn through the apparatus and the Kjeldahl flask is heated with the guarded Bunsen and the strength of flame adjusted so that at the end of 30 minutes about 15–20 cc. are left in the flask. This is merely a matter of a few trials. In this way the acetoacetic acid is broken down and the acetoacetic-acetone fraction distilled over with the formation

¹ The only reason for taking so small a volume is that in the investigations for which this method was employed serial collections of urine at short intervals were made and, in general, not more than 7 or 8 cc. urine were available; in this volume several observations had to be made.

of iodoform in the usual way. After 25 minutes' aeration and distillation, the current of air is increased and the flame turned off. After 30 minutes the receiver is disconnected, the delivery tube thoroughly washed down with distilled water, 5 cc. 20 % HCl are added and the contents titrated with freshly prepared $N/200$ thiosulphate using 1 % starch.

(It need hardly be emphasised that the blank of the apparatus and reagents must be accurately determined by repeated distillation. We have found an average blank of 1.5 cc. $N/200$ thiosulphate after 30 minutes' distillation.)

The volume in the Kjeldahl flask is now made up again to 40 cc. and another receiver containing the same quantities of iodine, etc. is connected. The flame is lowered a little by means of the screw clip and the $K_2Cr_2O_7-H_2SO_4$ mixture run in from the dropping funnel at a rate of about 1 drop every 2 or 3 seconds. Distillation and aeration are continued for 30 minutes when the receiver is disconnected and titrated as before. 1 cc. $N/50$ iodine = 0.193 mg. acetone = 0.50 mg. β -hydroxybutyric acid (70 % yield).

Notes on the Method.

1. The necessity of preliminary precipitation with the basic lead acetate solution, etc., at least in the estimation of β -hydroxybutyric acid, was shown by the following estimations in which the ketonic substances were determined with and without treatment with the lead. The figures given are volumes of thiosulphate required after distillation for 30 minutes into 10 cc. $N/50$ iodine, etc., as described above. I represents first distillation, II the oxidation of the hydroxy-acid.

10 cc. $N/50$ iodine required 39.6 cc. thiosulphate solution. Average blank for apparatus etc. 38.3 cc. thiosulphate solution.

Urine	With precipitation		Without precipitation	
	I	II	I	II
1	31.25	33.5	31.0	29.0
2	31.2	33.48	31.0	29.0
3	30.8	33.4	30.5	29.4
4	27.4	30.2	27.6	24.85
5	27.9	31.5	27.5	27.6

It is seen that the figures obtained by the two methods under I are very close to one another but this is not the case with those under II; that is to say, that without precipitation higher yields in " β -hydroxybutyric acid" will be obtained. The differences are as much as 4 or 5 cc. of thiosulphate which, assuming a yield of 70 %, would correspond to 0.5–0.6 mg. of β -hydroxybutyric acid.

This seems to us to be sufficient evidence of the necessity of preliminary precipitation.

2. Comparing the above method with Van Slyke's method we obtained the following results using diabetic urines. Acetoacetic acid and acetone only were estimated.

Urine	Percentage acetoacetic acid and acetone	
	Van Slyke mg.	Above method mg.
1	56.8	52.0
2	12.4	12.6
3	39.6	43.7
4	50.8	55.7

The general agreement is fair considering the widely different procedures.

3. The following experiments were carried out to find the yield of β -hydroxybutyric acid using the above method. For this purpose standard solutions of CaZn hydroxybutyrate were employed.

The CaZn salt was obtained as follows:

10 g. β -hydroxybutyric acid (Kahlbaum) was dissolved in 200 cc. distilled water and divided into two equal portions. To one portion excess of pure CaCO_3 was added and the mixture boiled for 30 minutes and then filtered. A clear brownish filtrate, consisting of calcium β -hydroxybutyrate, was obtained.

To the second portion of acid excess of freshly prepared well washed Zn(OH)_2 was added and the mixture boiled and filtered.

The two filtrates thus obtained were mixed and concentrated by gentle heat. The mixture was then poured slowly into excess of absolute alcohol, stirring vigorously. A finely divided white precipitate rapidly appeared. The alcoholic mixture was allowed to stand overnight and then centrifuged. The supernatant fluid was poured off, the remaining salt dissolved in a minimum of boiling water and again concentrated. Precipitation was carried out as before and the process repeated three times.

The pure white double salt thus obtained was placed in a desiccator and finally dried, by gentle heat, in a hot air oven to constant weight.

Analysis of the salt was carried out by incineration, the formula

being assumed, and it was found to be pure.

517.696 g. of the double salt are equivalent to 416.288 g. of β -hydroxybutyric acid.

A solution containing 0.1969 g. of the salt in 200 cc. distilled water was prepared; 1 cc. therefore containing 0.9845 mg. of the salt and being equivalent to 0.7915 mg. β -hydroxybutyric acid.

Yield of β -hydroxybutyric acid from urine using the method described.

In each estimation a different urine was used and a control carried out with the urine alone.

10 cc. urine and 10 cc. of above solution of CaZn hydroxybutyrate were mixed. 2 cc. of this mixture, after allowing for the content of the urine itself, contained the equivalent of 0.7915 mg. β -acid.

10 cc. of the mixture and 10 cc. distilled water were treated with 10 cc. basic lead acetate solution and 5 cc. ammonia, filtered, and 7 cc. of filtrate used for the estimation.

Blanks for the urine alone were obtained in each case by using 5 cc. urine, 15 cc. distilled water, 10 cc. basic lead acetate and 5 cc. ammonia and estimating in 7 cc. filtrate.

In this way the following yields were obtained:

Urine	Theoretical mg.	Amount found mg.
1	0.7915	0.50
2		0.49
3		0.58
4		0.59
5		0.52
6		0.57
7		0.54
8		0.53
9		0.49
10		0.51
11		0.53

The average recovery was thus 0.53 mg., i.e. 67.2 %.

Similar experiments were carried out using volumes to give theoretical yields of 1.583 mg. β -hydroxybutyric acid, with the following results:

Urine	Theoretical mg.	Amount found mg.
1	1.583	1.14
2		1.13
3		1.11
4		1.18
5		1.18
6		1.18
7		1.18

The average recovery was thus 1.16, i.e. 73.3 %.

A fresh solution of CaZn β -hydroxybutyrate was now prepared containing 1.7527 mg. β -hydroxybutyric acid (2.18 mg. salt) per cc. and estimations carried out as before:

Urine	Theoretical mg.	Amount found mg.
1	1.7527	1.270
2		1.23
3		1.29
4		1.30

The average recovery was thus 1.27, i.e. 72.5 %.

SUMMARY.

1. A method of estimating the ketonic substances in small volumes of urine is described, which yields results comparable to those given by other methods using larger volumes.

2. The estimation of β -hydroxybutyric acid by this method was investigated with the result that for quantities less than 1 mg. per cc. the yield was from 65–68 %, and for quantities between 1 and 2 mg. per cc. it was between 70 and 75 %.

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