CLXV. VAN SLYKE'S METHOD OF DETERMINATION OF ACETONE BODIES APPLIED TO SMALL VOLUMES OF BLOOD AND URINE.

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IN the method described below the proportions of the various reacting substances have been adjusted with the help of data given by Van Slyke [1917; Van Slyke and Fitz, 1917], and the strengths of solutions have been so arranged that the volumes are easily and accurately measured. The mercury-acetone precipitate is titrated, minor improvements being made in the technique, and a table is supplied to facilitate calculations. For a single estimation 0.5 cc. of blood or 1 cc. of urine is needed. It is possible therefore to make determinations on capillary blood obtained by skin puncture.

The writer has made parallel determinations with Van Slyke's original technique and the micro-technique described below on diabetic blood and urine, on known aqueous solutions of pure β -hydroxybutyric acid and acetone, and on normal blood to which known amounts of β -hydroxybutyric acid and acetone had been added. He finds that if the acetone body content is of the order of that found in severe ketosis (0.1 % reckoned as acetone), the micro-technique gives fully as accurate results as the original gravimetric technique of Van Slyke. In lesser grades of ketosis, the micro-technique is less accurate than the original technique, but it gives results of value in clinical work where only relatively gross variations in the content of acetone bodies are of significance. It gives an approximate measure of the acetone-body content of blood and urine even in health.

Apparatus and reagents.

The blood or urine is measured with an accurate Ostwald pipette. A reflux condenser with a straight inner tube of 8 mm. diameter is required for the oxidation. For the titration of the mercury-acetone precipitate, a 5 cc. micro-burette with a fine tip so that each drop is 0.02 cc. or less, and a dark blue tile are needed.

The acid mercuric sulphate solution is prepared by dissolving 45.6 g. of pure red oxide of mercury in one litre of 2.5 *N* sulphuric acid. The *M*/20 mercuric chloride solution is made by dissolving 13.576 g. of the A.R. salt in

water and diluting to 1 litre; standardisation by the sulphide method as described by Van Slyke is unnecessary. The 3.3 % potassium iodide solution is checked against the $M/20$ mercuric chloride solution each time a test is made (see later).

With every new batch of reagents a blank determination must be made, using water instead of blood or urine, to test the purity of the chemicals.

Removal of interfering substances.

Proteins *etc.* are removed from blood as follows. 0.5 cc. of whole blood is laked in 20 cc. of distilled water in a 25 cc. volumetric flask. 3 cc. of the acid mercuric sulphate solution are added, the volume is made up to 25 cc., and the flask is inverted several times to mix. After standing for 15 minutes the flask is again inverted and the contents are filtered.

Glucose¹ and other interfering substances are removed from urine as follows. In a stoppered graduated tube or cylinder 4 cc. of water, 1 cc. of urine, 2 cc. of 20 % copper sulphate, and 2 cc. of 10 % calcium hydroxide suspension are mixed. After a few minutes the reaction is tested with litmus, and if it is still acid, more of the calcium hydroxide suspension is added drop by drop until the reaction is alkaline. The mixture is then made up to 10 cc., allowed to stand for 30 minutes and filtered.

Oxidation of β -hydroxybutyric acid and separation of the mercury-acetone compound.

In a 100 cc. conical flask are placed 20 cc. of blood-filtrate, 3 cc. of 14*N* sulphuric acid, and 3 cc. of the acid mercuric sulphate solution, or 14 cc. of water, 4 cc. of urine-filtrate, 3 cc. of 14*N* sulphuric acid, and 5 cc. of the acid mercuric sulphate solution.

The flask is connected with the reflux condenser and the flame turned on. When the contents have been brought to the boil, 2 cc. of 2 % potassium dichromate are run down the condensing tube. Gentle ebullition is maintained for exactly 30 minutes.

After thorough cooling, the contents of the flask are filtered through a 9 cm. retentive paper (*e.g.* Whatman No. 42) without suction, and the precipitate is washed free from the reagents with water. Five washings usually suffice. The precipitate tends to float to the surface, wherefore a funnel should be selected which is a little smaller than the paper. (An alundum crucible may be used for the filtration.)

Titration of the mercury in the mercury-acetone compound.

Into a beaker of about 10 cm. diameter, 5 cc. of *N* hydrochloric acid are measured. The filter-paper is unfolded and dropped into the acid. The beaker is heated gently until the precipitate has dissolved, and then cooled to room

¹ The urine must be diluted if it contains more than 8 % of glucose.

temperature. 2 cc. of 40 % sodium acetate, one or two drops of 10 % sodium sulphite and 5 cc. of 3.3 % potassium iodide are added in the above order.

The excess of potassium iodide is determined by titration with $M/20$ mercuric chloride, the latter being added drop by drop until, when viewed against a dark blue tile, a reddish precipitate persists. The filter-paper may be lifted out just before the end-point is reached.

The sulphite is added to reduce any chromate derived from the mercury-acetone compound. Otherwise iodine is liberated from iodide by the chromate, thus introducing an error of about 7 %.

A blank titration of the potassium iodide solution against the $M/20$ mercuric chloride solution must be made each time an estimation of acetone bodies is undertaken. The same pipette should be used for measuring the 5 cc. of KI solution in both blank and test titrations. The blank titration is performed as follows. In a beaker are placed 5 cc. of N HCl, 2 cc. of 40 % sodium acetate, one or two drops of 10 % sodium sulphite (as in the test titration), and 5 cc. of 3.3 % potassium iodide in the order given. The $M/20$ mercuric chloride is run in until the red precipitate persists.

It is important that the potassium iodide should be checked against the $M/20$ mercuric chloride in the above way, because the end-point is affected by the p_H of the reaction mixture, slightly more $HgCl_2$ being required for the titration of a given volume of KI solution with increasing acidity. Using 3.3 % KI and the above technique, about 4.8 cc. of $M/20$ $HgCl_2$ are required to complete the blank titration.

Differential estimation of acetone bodies.

The above description applies when all the acetone bodies are to be estimated together.

For acetone *plus* acetoacetic acid, the same procedure is adopted except that the amount of $14N$ sulphuric acid is reduced to 2 cc. and the potassium dichromate is omitted. The filtrate from the mercury-acetone precipitate is preserved separately from the washings for the subsequent determination of β -hydroxybutyric acid.

For this purpose, the filtrate is measured in a cylinder and transferred to a 100 cc. conical flask. The filter-flask and cylinder are then washed out with water sufficient to make a total of 25 cc., and the washings are also transferred to the conical flask. After adding 1 cc. of $14N$ sulphuric acid, the conical flask is fitted to the condenser, and the contents are heated to boiling-point. The subsequent treatment, including the addition of the 2 cc. of 2 % potassium dichromate, is exactly as described for the determination of total acetone bodies.

This procedure is necessary for blood of which, moreover, the quantity available is often too limited to allow of a separate determination of β -hydroxybutyric acid. It may of course also be applied to urine. A separate estima-

tion of β -hydroxybutyric acid, after acidification and boiling off the volatile acetone bodies, is however more satisfactory. The procedure is as follows.

To 14 cc. of water and 4 cc. of urine-filtrate, 1 cc. of 14*N* sulphuric acid is added. The mixture is boiled for 10 minutes and cooled. The volume is then readjusted to the original by the addition of water. The remaining 2 cc. of 14*N* sulphuric acid and 5 cc. of acid HgSO_4 are then added, and the determination is completed as described for total acetone bodies.

Calculation.

In the blank titration the 5 cc. of KI solution are balanced by the added $M/20$ HgCl_2 . In the test titration a portion of the 5 cc. of KI solution has combined with the mercuric chloride formed by solution in hydrochloric acid of the mercury contained in the mercury-acetone precipitate: the rest of the 5 cc. of KI solution is balanced by the $M/20$ HgCl_2 required to complete the titration. Therefore the difference between the blank titration and the test titration (*i.e.* the $M/20$ HgCl_2 deficiency) measures the quantity of acetone bodies in the amount of blood- or urine-filtrate employed.

Acetone. The mercury-acetone compound contains 77 % mercury and therefore 1 cc. of $M/20$ HgCl_2 deficiency corresponds to 13 mg. of the mercury-acetone precipitate (*i.e.* 10 mg. mercury).

It is found that 1 mg. of acetone gives 20 mg. of precipitate, *i.e.* each mg. of precipitate corresponds to 0.05 mg. acetone.

From this it follows that 1 cc. of HgCl_2 deficiency represents 13×0.05 mg. of acetone; using a blood-sample of 0.4 cc. this means an acetone concentration of $13 \times 0.05 \times 250$ or **162.5 mg./100 cc.**

β -Hydroxybutyric acid. In the case of β -hydroxybutyric acid it is found that 1 mg. of the acid yields 6.7 mg. of precipitate.

But 104 g. (1 g.-mol.) of β -hydroxybutyric acid should yield 58 g. (1 g.-mol.) of acetone.

Whence 1 mg. of β -hydroxybutyric acid should yield $\frac{58}{104} \times 20$ mg. of precipitate.

This means that under the conditions of oxidation employed the yield of acetone from β -hydroxybutyric acid is $\frac{6.7 \times 104}{58 \times 20} \times 100$ or 60 % of the theoretical; *i.e.* 1 g.-mol. of β -hydroxybutyric acid yields 0.6 g.-mol. of acetone.

Expressed numerically:

104 g. β -hydroxybutyric acid yield 0.6×58 g. acetone.

$\therefore$ 1 mg. acetone produced corresponds with $\frac{104}{0.6 \times 58}$ mg. β -hydroxybutyric acid but 1 mg. acetone produced corresponds with 20 mg. ppt. and $\therefore$ with $\frac{20}{13}$ cc. HgCl_2 deficit.

$\therefore$ 1 cc. HgCl_2 deficit represents $\frac{13}{20} \times \frac{104}{0.6 \times 58}$ or 1.942 mg. β -hydroxybutyric acid in the sample, *i.e.* 486 mg. β -hydroxybutyric acid per 100 cc. blood if the sample is 0.4 cc.

Recalculating as acetone this figure becomes $486 \times \frac{58}{104}$ or **270.8** mg./100 cc.

Total acetone substances. For the purpose of this calculation it is assumed that the mixture being analysed contains β -hydroxybutyric acid and acetone in the molecular ratio 3 : 1.

Now 3 g.-mols. of β -hydroxybutyric acid will yield 3×0.6 or 1.8 g.-mols. of acetone; *i.e.* 4 g.-mols. of the mixture will yield $1.8 + 1$ or 2.8 g.-mols. of acetone.

Expressed numerically:

$(3 \times 104 + 58)$ g. mixture yield 2.8×58 g. acetone,

whence 1 mg. of acetone produced represents $\frac{370}{2.8 \times 58}$ mg. mixture.

$\therefore$ 1 cc. of HgCl_2 deficit represents $\frac{13}{20} \times \frac{370}{2.8 \times 58} = 1.481$ mg. mixture in sample, or 1 cc. of HgCl_2 deficit represents $1.481 \times 250 = 370$ mg. mixture/100 cc. blood (the sample being 0.4 cc.).

In order to convert to acetone the last figure must be multiplied by $\frac{4 \times 58}{370}$, giving **232.1** mg./100 cc. blood as acetone. Figures worked out on this basis for HgCl_2 deficits up to 1 cc. using 0.4 cc. samples are given in Table I.

Table I. *Relationship between the M/20 HgCl_2 deficiency and concentration of acetone bodies in blood or urine.*

(Sample = 0.4 cc.)

cc. M/20 HgCl_2 defi- ciency	Total acetone bodies as mg. of acetone per 100 cc.					Acetone and acetoacetic acid as mg. of acetone per 100 cc.					β -Hydroxybutyric acid as mg. of acetone per 100 cc.				
	.00	.02	.04	.06	.08	.00	.02	.04	.06	.08	.00	.02	.04	.06	.08
0.00	0	5	9	14	19	0	3	7	10	13	0	5	11	16	22
0.10	23	28	32	37	42	16	20	23	26	29	27	32	38	43	49
0.20	46	51	56	60	65	33	36	39	42	46	54	60	65	70	76
0.30	70	74	79	84	88	49	52	55	59	62	81	87	92	97	103
0.40	93	97	102	106	111	65	68	72	75	78	108	114	119	125	130
0.50	116	120	125	130	135	81	85	88	91	94	135	141	146	152	157
0.60	139	144	149	153	158	98	101	104	107	111	162	168	173	179	184
0.70	162	167	172	176	181	114	117	120	124	127	190	195	200	206	211
0.80	186	190	195	200	204	130	133	137	140	143	217	222	227	233	238
0.90	209	214	218	223	227	146	150	153	156	159	244	249	255	260	265
	1 cc. = 232.1					1 cc. = 162.5					1 cc. = 270.8				

The clinical significance of estimation of acetone bodies in blood.

At the present date when doubt has been thrown on the view that the ketone bodies are the most important factor in the causation of diabetic coma, a simple method of estimating the degree of ketonaemia is likely to be useful both for the investigation of the aetiology of coma and for the treatment of individual cases. It is well known that when the diminution of alkali reserve does not run parallel with the concentration of ketone bodies in blood, the clinical condition (severity of coma *etc.*) accords with the former rather than with the latter and in such cases beneficial effects have been obtained by

administering alkalis. It would be worth while therefore to estimate the degree of ketonaemia in order to decide whether insulin should be pushed further or a trial given to alkalis. The degree of ketonuria may be of no help since it is now definitely known that if the kidneys have been damaged they may fail to excrete ketone bodies.

In view of the finding of Starr and Fitz [1924] that in several cases of severe diabetes investigated by them there was evidence of the presence of some organic acid other than the known keto-acids, it would be interesting to calculate the sum of the alkali reserve as determined and the diminution of CO_2 -combining power of blood attributable to the fixation of base by the keto-acids. One molecule of a keto-acid may be taken to displace one molecule of CO_2 from one molecule of NaHCO_3 according to the formula

Thus keto-acids corresponding to 58.05 mg. acetone would displace 44 mg. CO_2 , i.e. 22.4 cc. at N.T.P. Therefore the concentration (as mg. acetone per 100 cc.) of acetone bodies in blood multiplied by $\frac{22.4}{58.05}$, i.e. 0.385, will give the diminution of CO_2 -combining power due to the presence of the keto-acids. When this is added to the alkali reserve as determined, a normal figure should be obtained if the acidosis is principally caused by acetone bodies. Conversely if a figure much lower than normal is obtained, there is presumptive evidence of the presence of other organic acids in the blood. In such cases perhaps too much reliance on insulin would not be justified and administration of alkalis might be of help. (The reasoning employed here is essentially the same as that of Starr and Fitz, only expressed in a different way.)

During recovery from coma acetone bodies in blood fluctuate very widely. It is possible that the sudden great rises met with, while the clinical condition is steadily improving, are due to the administration of glucose. On several occasions, while studying blood-sugar curves in diabetics, the writer has observed a marked rise in ketonaemia and ketonuria along with the rise of blood-sugar. (The ketone bodies fall however more rapidly than blood-sugar.)

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