CXLVI. THE DETERMINATION OF PYRUVIC ACID.

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(*Received May 25th, 1928.*)

THE methods employed for the estimation of pyruvic acid are largely based upon the reaction of its carbonyl group with phenylhydrazine. Its estimation in complex biological fluids where there are other compounds which react likewise is therefore difficult and, particularly when pyruvic acid exists in small quantities amidst these compounds, the estimation is unreliable even when controls are run. This difficulty was greatly felt in some of our investigations, and it was thought worth while to study some of the existing methods which could be satisfactorily employed as such or modified for our purpose.

The production of an intense blue colour by sodium nitroprusside and ammonia, especially in the presence of a little acetic acid, has been claimed to be specific for pyruvic acid and unaffected by acetaldehyde.

The precipitation of pyruvic acid as hydrazone by excess of phenylhydrazine followed by the determination of the excess phenylhydrazine has been the subject of considerable study [Smedley-MacLean, 1913; Simon and Piaux, 1924].

The reduction of pyruvic to lactic acid by zinc and hydrochloric acid and the subsequent estimation of lactic acid according to the method of Fürth and Charnass has been adopted by Lieben [1923].

The oxidation of pyruvic to acetic acid by a slight excess of hydrogen peroxide has been shown to be quantitative by Holleman [1904], and potassium dichromate has been employed similarly and the excess dichromate estimated.

As it is not the purpose of this paper to give the results of the comparative study of these methods, it will be sufficient to say that none of them as such was found suitable in our experiments, and all required some modifications before they could be employed.

The necessary conditions for a method which would be suitable to our requirements were that it should be applicable to small quantities of the acid, usually between 0·5 and 15 mg., and in solutions of very low concentration, *i.e.* 0·1 % to 0·05 %.

The method finally developed is a modification of Lieben's technique, and is based upon the following reactions:

$$CH_3.CO.COOH + 2H = CH_3.CHOH.COOH \quad \text{.....(i).}$$

$$CH_3.CHOH.COOH + O = CH_3.CHO + CO_2 + H_2O \quad \text{.....(ii).}$$

The reduction of pyruvic to lactic acid by zinc and hydrochloric acid has been shown to be quantitative by Lieben. The lactic acid was estimated according to Fürth and Charnass. A micro-adaptation of Fürth and Charnass's method, with small quantities of pyruvic acid reduced by zinc and hydrochloric acid, gave very irregular results, due probably to very slight but irregular decomposition of pyruvic acid when boiled for a long time with 10 % HCl. With higher concentrations of pyruvic acid the results are of the same degree of accuracy as those of Lieben, and, with an empirical factor for lactic acid, its equivalent of pyruvic acid can be accurately calculated. Some of the results obtained according to the micro-adaptation of Fürth and Charnass's method of oxidation, and for larger amounts of acid according to the original method, are given in Tables I and II respectively. In all these cases, however, the titration of bound aldehyde was made according to Clausen [1922].

Table I.

Different amounts of pyruvic acid were reduced by boiling with 50 cc. of 10 % HCl and 0·5 to 0·75 g. of zinc for 2¾ hours.

Pyruvic acid taken (mg.)	0·68	1·36	1·36	2·04	2·04	3·40	3·40	2·72
Found (%)	79·4	85·3	81·6	85·3	82·9	86·5	82·9	83·1
Pyruvic acid taken (mg.)	2·72	6·80	6·80	10·20	10·20	13·60	13·60	
Found (%)	84·6	85·5	82·0	86·2	88·8	89·2	87·1	

According to the same method Clausen finds with lactic acid a yield on an average of approximately 92 %, about the same as that found for larger quantities. For comparison some results of the estimation of larger quantities of pyruvic acid according to the original method of Fürth and Charnass are given in Table II.

Table II.

Different amounts of pyruvic acid were reduced by boiling with 100 cc. of 10 % HCl and 2 g. of zinc for 2¾ hours.

Pyruvic acid taken (mg.)	12·14	12·14	24·28	24·28	36·42	36·42	48·56
Found (%)	86·9	85·8	86·7	90·2	88·3	87·9	88·5
Pyruvic acid taken (mg.)	48·56	60·7	60·7	121·4	121·4	182·1	182·1
Found (%)	89·9	88·4	90·1	89·56	91·6	89·6	92·2

The values for pyruvic acid computed according to the empirical factor of Fürth and Charnass give results accurate to within about ± 3 %.

The irregular results obtained when small amounts of pyruvic acid are estimated were at first supposed to be due to the length of time during which they were in contact with boiling acid, and unsuccessful attempts were made to find a time during which a maximum and constant yield was obtained.

In view of this irregularity, reduction at low temperatures was tried and the use of a zinc-copper couple in sulphuric acid solution was found satisfactory. The oxidation of lactic acid was carried out by a slight modification of a recent method due to Friedmann *et al.* [1927]. In this case the yield was lower than with other methods (about 80 %) but it was quite constant within a large range of pyruvic acid concentrations.

The apparatus used by us is shown in Fig. 1 and is much simpler than that of Friedmann. The transference of aldehyde to the receiver is facilitated by passing carbon dioxide from a cylinder through the apparatus. The gas is passed through a long column of saturated sodium bisulphite solution and then through water, which is frequently changed, before it enters the reaction vessel. The yield of aldehyde has been found to depend upon the rate of aeration also, and this can be effectively controlled.

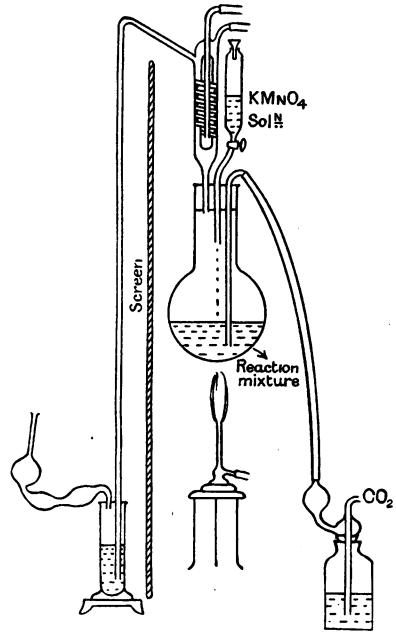

Fig. 1.

THE ESTIMATION OF PYRUVIC ACID IN PURE SOLUTIONS.

1 to 5 cc. of solution containing 0·25 to 15 mg. of pure pyruvic acid are treated with 50 cc. of 17·5 % H_2SO_4 and 0·5 to 1 g. zinc together with 1 cc. of 10 % copper sulphate solution. After an hour the solution is filtered into the reaction flask (a 500 cc. Kjeldahl flask) and neutralised slowly with 60 % NaOH added drop by drop, dimethylaminoazobenzene being the indicator. 10 cc. of 10 *N* H_2SO_4 containing 0·1 *N* $MnSO_4$ are then added and the oxidation is carried out with 0·01 or 0·005 *N* permanganate, the bound aldehyde being then titrated according to Clausen's method. The use of hydroxylamine to catch the acetaldehyde, as suggested by Leone and Tafuri [1925], was tried, but the end-point was not very sharp, and in all further work Clausen's method was adopted. Some of the results of estimations of pyruvic acid in pure solution with different quantities of the acid are given in Table III. A few experiments were also conducted in which the oxidation of the reduced

pyruvic acid was carried out with sulphuric acid. The yield of aldehyde obtained when 50 % sulphuric acid at 140° was used according to Clausen showed that more drastic treatment was required, namely increase of the temperature to 160°–170° and the use of 60 % acid, as had been found by Hill, Long and Lupton [1924] and Ronzoni and Laurence [1927]. Some of the results obtained are given in Table IV.

Table III. *Analyses of pyruvic acid in pure solutions.*

Pyruvic acid taken (mg.)	16·36	16·36	13·62	13·62	8·18	8·18	6·10	6·10
Found (%)*	99·6	99·4	101·9	97·8	99·3	99·3	100·6	100·6
Pyruvic acid used (mg.)	3·44	2·44	2·44	1·22	1·22	0·61	0·61	0·305
Found (%)*	97·7	97·6	97·6	102·3	98·4	103·2	100·0	100·0

* Using factor (see p. 1173).

Table IV. *Comparison of the permanganate and sulphuric acid methods of oxidation.*

Pyruvic acid taken (mg.)	8·43	8·43	5·06	3·37	0·61	0·305	1·69
Permanganate method, found (%)	80·4	82·3	80·3	78·9	80·3	82·6	76·3
Sulphuric acid method, found (%)	80·9	80·7	81·0	82·8	78·7	78·7	78·0

The results of the estimation of pyruvic acid in pure solution according to both the methods of oxidation, showed that there is a yield of 80 % aldehyde, constant over large variations in the quantities of pyruvic acid used. This does not appear to be due to a factor in the oxidation of lactic acid since pure zinc lactate solutions could be determined by the same method with an accuracy of 96·2 to 97·9 %.

The actual losses of pyruvic acid appear to occur during the initial reduction process, probably due to the formation, along with lactic acid, of products which do not yield bisulphite-binding compounds during the subsequent oxidation. Ronzoni and Laurence [1927], in connection with their experiments on the loss of lactic acid in solution, drew attention to an early observation of Dakin, who found that lactic acid solutions on standing yielded methylglyoxal. They moreover found that a solution of zinc lactate on standing for several days gave smaller and smaller yields of aldehyde day by day until after 4 days the yield stood constant at about 84 % for several weeks. This fall was noticed even when the solution was sterile and in the presence of mercuric chloride. We ourselves have observed such a loss of aldehyde, but the fall, though considerable, was not so rapid as that found by Ronzoni and Laurence. It was also found that zinc lactate solution, treated in the same way as pyruvic acid in our experiments, gave lower yields of aldehyde.

Another source of loss of lactic acid before oxidation appears to be due to the fact that it is kept in contact with sulphuric acid, and this loss increases with the time of contact. 23·3 mg. of zinc lactate were made up to 100 cc. with 20 % sulphuric acid. 20 cc. of this solution were taken at intervals of $1\frac{1}{2}$ hours and the lactic acid was estimated. The results are given in Table V.

Table V.

Time during which lactic acid was in contact with sulphuric acid (hrs.)	0	1½	3	4½	6
Aldehyde yield (%)	97·6	96·5	95·4	94·2	93·1

Taking all these observations into consideration, we are led to imagine that the small yield of aldehyde in our case may be due to the same cause as that which lessened the yield of aldehyde with lactic acid solution on standing. The possibility exists of the formation of methylglyoxal from pyruvic acid if the carboxyl group is attacked, along with the carbonyl group, through the vigorous reducing action of the zinc-copper couple, although the formation of methylglyoxal is not known to occur from reduction of pyruvic acid in practice.

Since in the estimation of pyruvic acid by this method the errors due to reduction or oxidation are quite regular over a large range of concentration of pyruvic acid, the particular drawback of a small yield of aldehyde and the consequent necessity of using a large factor to compute the actual amount, cannot materially diminish the value of the method for the estimation of small quantities of pyruvic acid. This is shown by calculating the probable error of a single determination by the formula

$$e = \pm 0{\cdot}6745 \sqrt{\frac{d_1^2 + d_2^2 + \dots d_n^2}{n-1}},$$

where d represents the deviation of an observation from the mean of the series and n the number of observations. The probable error of the mean (e_m) is given by the equation

$$e_m = \frac{e}{\sqrt{n}}.$$

The probable error from a series of 15 determinations on the same quantity of pyruvic acid is shown in Table VI, and also the probable error of the mean.

Table VI.

10·18 mg. of pyruvic acid were reduced by 40 cc. of 17·5 % H_2SO_4 plus 0·75 g. Zn and a trace of copper sulphate.

Amount of acid taken (mg.)	Amount found (mg.)	Deviation from the mean (d)	d^2
10·18	8·10	−0·04	0·0016
	8·18	+0·04	0·0016
	8·04	−0·10	0·0100
	8·10	−0·04	0·0016
	8·03	−0·11	0·0121
	7·92	−0·22	0·0484
	8·27	+0·13	0·0169
	7·90	−0·24	0·0576
	8·06	−0·08	0·0064
	7·95	−0·19	0·0361
	8·14	+0·00	0·0000
	8·00	−0·14	0·0196
	8·29	+0·15	0·0225
	8·06	−0·08	0·0064
	8·16	+0·02	0·0004

Mean = 8·14
$\Sigma d^2 = 0{\cdot}2412$
therefore $e = 0{\cdot}0885$
and the error of mean (e_m) = 0·0237.

The probable error of a single determination is, therefore, about 1·1 %. The mean of this series is very nearly the same as that obtained from the determination with different quantities of pyruvic acid (Table III).

Thus, if an empirical factor covering the regular error be taken, the exact amount of pyruvic acid can be computed. This factor works out to about 20 %, *i.e.* 1 cc. *N*/10 iodine represents 5·5 mg. pyruvic acid. The values calculated on this empirical basis for various quantities of pyruvic acid have been given in Table III.

Application of the proposed method to biological fluids.

The question of the specificity of the reactions upon which the proposed method of estimation is based arises when the method is applied to biological fluids. It will be seen that not only hydroxy-acids, carbohydrates and acetone but also lactic acid and acetaldehyde interfere with the determination and must be removed from the solution before pyruvic acid is estimated. The separation of pyruvic acid from other bisulphite-binding compounds is not easy, and the results of titration cannot be taken as specific unless acetaldehyde from pyruvic acid only be taken as a measure of that acid. During the usual processes for the removal of carbohydrates or proteins from biological materials, these interfering agents are not removed. It is found that pyruvic acid bound by sodium bisulphite is not extractable by ether, whereas lactic acid and other hydroxy-acids are easily extracted. Therefore the pyruvic acid may be extracted along with lactic acid and other interfering substances by a preliminary ether extraction which separates them from carbohydrates also. The ether extract with a small quantity of sodium bisulphite solution is again extracted with ether, when the pyruvic acid, which is combined with bisulphite, is separated from lactic acid, β-hydroxybutyric acid and other interfering substances.

Thus, since ether extraction was used for the separation of pyruvic acid from carbohydrates and lactic acid, there was no necessity to adopt Van Slyke's method [1917] for the removal of carbohydrates from solution. It was moreover found that considerable amounts of pyruvic acid are lost in that process.

We had therefore to study only the separation of proteins from dilute pyruvic acid solution. Experiments were carried out with caseinogen and haemoglobin solutions to which small quantities of pyruvic acid were added. In the case of body fluids, it is found that the method of Folin and Wu [1919] can be employed. For the separation of proteins from yeast culture solutions, precipitation by alcohol is sometimes preferable. The solution is rendered faintly acidic and 15 to 20 times its volume of 99 % alcohol added. The solution is kept overnight, filtered, and the precipitate washed with 98 % alcohol.

An aliquot portion of the solution after the precipitation of proteins, either by the method of Folin and Wu or by the use of alcohol, is neutralised and

evaporated under reduced pressure at 40° to 50° almost to dryness. The residue is then transferred with a small quantity of saturated ammonium sulphate solution to the ether extractor, the solution rendered acid to methyl red, and the pyruvic acid extracted. The ether is evaporated and the pyruvic acid is bound by sodium bisulphite, and the solution is extracted again with ether to remove lactic acid, etc. It is estimated as before and the exact amount calculated by means of the empirical factor.

Table VII. *Recovery of pyruvic acid from protein solutions.*

Pyruvic acid added (mg.)	Protein solution	Method of precipitation	Pyruvic acid recovered (%)
4·61	2 % haemoglobin	Folin and Wu	99·2
4·61	5 % ,,	,, ,,	98·9
4·61	2 % ,,	Alcohol	99·35
4·61	5 % ,,	,,	98·9
7·69	3 % ,,	Folin and Wu	99·45
7·69	3 % ,,	Alcohol	100·1
15·37	5 % ,,	Folin and Wu	99·48
15·37	5 % ,,	Alcohol	97·8
3·05	2 % caseinogen	Folin and Wu	98·4
3·05	2 % ,,	Alcohol	97·7
6·10	3 % ,,	Folin and Wu	98·5
6·10	3 % ,,	Alcohol	100·2
15·25	3 % ,,	Folin and Wu	99·2
15·25	3 % ,,	Alcohol	98·3
15·25	5 % ,,	Folin and Wu	97·9
15·25	5 % ,,	Alcohol	97·6

From such dilute solutions of pyruvic acid a recovery of 97 % is therefore possible after protein precipitation.

The estimation of pyruvic acid in biological fluids may therefore be carried out as follows. The solution, which should contain not more than 15 mg. of pyruvic acid during its reduction to lactic acid, is taken and the proteins separated by either of the processes detailed above. Suppose the solution contains 0·05 %, about 2 to 5 cc. of this is used for protein precipitation and the whole of the filtrate is taken up for subsequent processes. The filtrate is rendered neutral to litmus and evaporated under diminished pressure at 40° to 50°. In this process much of the preformed acetone, acetaldehyde or other volatile compounds, escapes. The substance is then transferred to a Clausen or Meyerhof extractor with a small quantity of saturated ammonium sulphate solution, rendered slightly acid and extracted with ether to separate the carbohydrates from the ether-extractable substances. The ether should have been distilled over bisulphite, as it sometimes contains bisulphite-binding compounds as impurities. The ether extract is evaporated to dryness and shaken up with a quantity of sodium bisulphite three or four times in excess of the expected amount of pyruvic acid. It is then transferred again to the ether extractor to separate pyruvic acid from lactic acid, β-hydroxybutyric acid, phenols, etc. The residue is transferred to a 100 cc. flask and the pyruvic acid reduced by sulphuric acid and zinc with a trace of copper. The lactic acid is estimated as already described.

The errors introduced by the presence of acetone bodies is considerably diminished by the two evaporations. But even then the error due to acetone formed during oxidation, or held tenaciously, can be determined after the removal of aldehyde by Schaffer's method [1908], as adopted by Clausen as a correction for his lactic acid determination. The titre from this distillation is subtracted from the previous titre, and after proper corrections are applied the pyruvic acid is calculated using as factor 1 cc. of $N/100$ iodine = 0·55 mg. pyruvic acid. In many cases, except where the determination is carried out with yeast culture solutions, this determination of acetone bodies after final iodine titration is unnecessary, as the error is small. The results of the recovery of pyruvic acid added in small quantities to biological fluids, such as the body fluids of the lac insect, sheep's blood and yeast culture solutions, are given in Table VIII. The probable error of a single determination in a series, calculated from a number of experiments as before, is shown in Table IX.

Table VIII. *Recovery of pyruvic acid added to biological fluids.*

Medium	Amount added (mg.)	Iodine titre corrected for blank and titre (cc.)	Control (cc.)	Amount recovered using the empirical factor	Error (%)
Sheep's blood, 5 cc.	1·51	5·5 (0·005 N)	0·10?	1·49	−1·3
	3·02	11·3 (0·005 N)	0·10?	3·08	+2·0
	6·04	11·15 (0·01 N)	0·10?	6·08	+0·7
Yeast culture solution, 5 cc.	1·51	5·65 (0·005 N)	0·25	1·49	−1·3
	3·02	11·12 (0·005 N)	0·25	2·99	−1·0
	6·04	10·96 (0·01 N)	0·25	5·89	−2·5
Body fluid of lac insect, 5 cc.	1·51	5·76 (0·005 N)	0·20	1·53	+1·3
	3·02	11·02 (0·005 N)	0·20	2·98	−1·5
	6·04	10·86 (0·01 N)	0·20	5·87	−2·9

Table IX.

4·48 mg. of pyruvic acid were added to 5 cc. of the body fluid of the lac insect.

Exp. no.	Amount of pyruvic acid calculated (mg.)	d	d^2
1	4·44	+0·05	0·0025
2	4·30	−0·09	0·0081
3	4·38	−0·01	0·0001
4	4·51	+0·12	0·0144
5	4·35	−0·04	0·0016
6	4·32	−0·07	0·0049
7	4·27	−0·12	0·0144
8	4·56	+0·17	0·0289
9	4·46	+0·07	0·0049
10	4·36	−0·03	0·0009

Mean = 4·39.
Probable error $e = 0·063$; *i.e.* about 1·4 %.

Summary.

1. A new method for the estimation of pyruvic acid in small quantities has been described which is claimed to be more specific than the existing methods when applied to biological fluids.

2. The probable error of a single determination has been determined.

Our grateful thanks are due to Prof. R. V. Norris, for the keen interest he has shown in the work.

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