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CONTENTS.

DR. M. O. FORSTER, F.R.S., CHAIRMAN OF EDITORIAL BOARD.

I.—CHARACTERISATION OF VERY SMALL QUANTITIES OF PROTEINS BY VAN SLYKE'S METHOD.*

By Nuggihalli Narayana and Mothnahalli Sreenivasaya.

In the course of our studies on animal and vegetable proteins, it became necessary to deal with very small quantities of substances, about 100 to 150 mg., a twentieth of the quantity usually employed for a macro-analysis. Thimann (*Biochem. J.*, 1926, **20**, 1190) has described a method for the determination of the Hausmann numbers in small quantities of proteins and we have extended the method for a more comprehensive characterisation of proteins by Van Slyke's method.

EXPERIMENTAL.

Digestion.—100 to 150 mg. of the protein were weighed out into a small Kjeldahl flask (4 × 30 cm.), fitted with a ground glass air condenser and digested for 36 hours with 10 c.c. of 20 per cent. hydrochloric acid. The acid was then removed by distillation in vacuo (20–25 mm.) at 40–45°. Soda-lime was placed in the receiver and a slow current of ammonia-free air was passed during the distillation to facilitate removal of the acid. A fresh quantity of ammonia-free water was added and the distillation repeated under similar conditions. The titratable acidity of the digest after distillation in the successive duplicates lay between very narrow limits. This fact is of great importance in connection with the subsequent precipitation of the hexone bases, which has been shown by Knaggs (*Biochem. J.*, 1923, **17**, 488) to depend on the concentration of the salts present in the digest of the amino-acids.

The syrupy mass was dissolved in ammonia-free water and made up to about 40 c.c. Two portions of 2 c.c. each were taken for total nitrogen, two of 10 c.c. each for the series of analyses and two of 5 c.c. each for the estimation of arginine.

Amide nitrogen.—Each of the 10 c.c. portions was neutralised with $N/2$ alkali till just pink to phenolphthalein and, after adding a drop

* Reprinted from *The Biochemical Journal*, 1928, **22**, 1135.

of the alkali in excess, the mixture was aerated for 2 hours at room temperature, air free from ammonia and carbon dioxide being passed. A few drops of amyl alcohol were added to prevent foaming. The ammonia was absorbed in 10 c.c. of $N/70$ alkali.

Melanin nitrogen.—The solution, after aeration, was carefully neutralised with $N/5$ hydrochloric acid and the melanin filtered through a small Jena glass filter covered with a thin layer of asbestos. The nitrogen in the precipitate was determined by the micro-Kjeldahl process.

Hexone bases.—The filtrate was evaporated on a water-bath to about 5 c.c., washed into a 20 c.c. stoppered centrifuge tube and made up to nearly 10 c.c. About 1 c.c. of concentrated hydrochloric acid was then added. The tube was plugged with cotton wool and autoclaved for 30 minutes at 25 lbs. pressure (Knaggs, *loc. cit.*); 3 c.c. of 25 per cent. phosphotungstic acid solution were then added and the tube was placed in a boiling water-bath until the precipitate had almost redissolved. After cooling, the tube was placed in the ice-chest for 36 hours. The precipitate was then centrifuged, and washed in the centrifuge thrice with ice-cold hydrochloric acid (1–10), using 3 c.c. each time. The clear centrifugate and the washings were passed through a small filter to recover any suspended particles. The combined precipitates were dissolved by the gradual addition of $N/2$ sodium hydroxide until the pink colour formed with phenolphthalein remained just permanent. The solution was made up to 25 c.c. and 5 c.c. were employed for total nitrogen and another 5 c.c. for amino-nitrogen and 10 c.c. for a micro-estimation of sulphur according to Pregl. From the sulphur-content the amount of cystine in the precipitate of the bases was calculated.

Arginine.—Arginine was estimated by arginase which was prepared from the liver of a ram. The enzyme preparation was found to be free from aminases and amidases which liberate ammonia from aminoacids and amides and its activity on a solution of arginine was determined; 97 to 98 per cent. of the arginine was found to be hydrolysed under the conditions which obtain in the accompanying experiments.

5 c.c. portions of the original amino-acid digest were neutralised with $N/2$ sodium hydroxide, ammonia was removed by aeration, as before and the P_H adjusted to 9·7 by adding a phosphate buffer. After addition of 0·5 c.c. of the enzyme extract and 1 c.c. of toluene, the reaction was allowed to proceed for 36 hours at 37°. Controls were run with 5 c.c. of water and 0·5 c.c. of the fresh enzyme extract. Urease

solution, corresponding to half a pellet of Dunning's preparation, was then added to the reaction mixture, the P_H being readjusted to 7. After 12 hours' reaction at 37° a saturated solution of potassium carbonate was added in slight excess and the ammonia estimated by aeration.

From a knowledge of the content of cystine, arginine and the non-amino-nitrogen in the basic fraction, the histidine and lysine contents were calculated.

Mono-amino-fraction.—The combined filtrates from the precipitation of the hexone bases were made up to 50 c.c. of which two 10 c.c. portions were used for total nitrogen and two 5 c.c. portions for amino-nitrogen. All ammonia distillations were carried out in the Parnas-Wagner modification of Pregl's micro-apparatus, and the amino-nitrogen determinations made with Van Slyke's micro-apparatus.

TABLE I.—CASEINOGEN.

Results expressed as percentages of total nitrogen.

—	(Micro) Narayana and Sreeniva-saya	(Macro) Narayana and Sreeniva-saya	Hoffman and Gortner (1924)	Dunn and Lewis (1921)	Van Slyke (1914)
Ammonia-N	9·90	10·12	10·20	10·49	10·27
Humin-N	1·16	1·86	1·51	2·13	1·28
Basic N :—					
Arginine-N	9·89	9·01	9·20	7·42	7·41
Histidine-N	3·88	4·16	6·26	6·01	6·21
Cystine-N	0·96	0·68	1·05	0·48	0·21
Lysine-N	7·67	8·12	8·49	9·09	10·30
N in filtrate from bases :—					
Amino-N	59·20	57·30	54·12	58·78	55·81
Non-amino-N ...	9·02	9·10	8·76	5·93	7·13
Total ...	101·68	100·35	99·59	100·33	98·61

TABLE II.—GELATIN.

Results expressed as percentages of total nitrogen.

	(Micro) Narayana and Sreenivasaya	(Macro) Narayana and Sreenivasaya	(Micro) Thimann (1926)
Ammonia-N	1·84	1·70	2·60
Humin-N	Trace.	0·56	0·00
Basic N :—	22·61	25·04	17·60
Arginine-N	15·87	...	...
Histidine-N	2·74	...	...
Lysine-N	4·00	...	...
N in filtrate from bases .—	76·2	71·06	79·60
Amino-N	57·40	...	...
Non-amino-N	18·80	...	...
Total ...	100·65	98·36	99·8

SUMMARY.

A method for the characterisation of very small quantities of proteins by Van Slyke's method has been described.

Our grateful thanks are due to Prof. R. V. Norris for the keen interest he has evinced during the course of this work.

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II.—THE DETERMINATION OF PYRUVIC ACID.*

By Basettihalli Hanumantha Rao Krishna and Mothnahalli Sreenivasaya.

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, *Biochem. J.*, 1913, **7**, 611, Simon and Piaux, *Bull. Soc. Chim. Biol.*, 1924, **6**, 477).

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 (*Biochem. Z.*, 1923, **135**, 240).

The oxidation of pyruvic to acetic acid by a slight excess of hydrogen peroxide has been shown to be quantitative by Holleman *Rec. Trav. Chim.*, 1904, **23**, 147), 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 per cent.

* Reprinted from *The Biochemical Journal*, 1928, **22**, 1169.

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

$$CH_3{\cdot}CO{\cdot}CO_2H + 2H = CH_3{\cdot}CH(OH){\cdot}CO_2H \quad \text{.....(i).}$$

$$CH_3{\cdot}CH(OH){\cdot}CO_2H + O = CH_3{\cdot}CHO + CO_2 + H_2O \quad \text{.....(ii).}$$

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 per cent. hydrochloric acid. 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 (*J. Biol. Chem.*, 1922, **52**, 263).

TABLE I.

Different amounts of pyruvic acid were reduced by boiling with 50 *c.c. of* 10 *per cent. 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, per cent.	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, per cent.	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 per cent., 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 *c.c.* 10 *per cent. 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, per cent.	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, per cent.	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 per cent.

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.* (*J. Biol. Chem.*, 1927, **73**, 335). In this case the yield was lower than with other methods (about 80 per cent.) 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

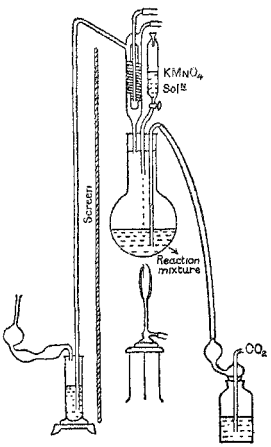

FIG. 1.

THE ESTIMATION OF PYRUVIC ACID IN PURE SOLUTIONS.

been found to depend upon the rate of aeration also, and this can be effectively controlled.

1 to 5 c.c. of solution containing 0·25 to 15 mg. of pure pyruvic acid are treated with 50 c.c. of 17·5 per cent. H_2SO_4 and 0·5 to 1 g. zinc together with 1 c.c. of 10 per cent. copper sulphate solution. After an hour the solution is filtered into the reaction flask (a 500 c.c. Kjeldahl flask) and neutralised slowly with 60 per cent. NaOH added drop by drop, dimethylaminoazobenzene being the indicator. 10 c.c. of 10N H_2SO_4 containing 0·1N $MnSO_4$ 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 (*Ann. Chim. Applic.*, 1925, **15**, 206), 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 per cent. 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 per cent. acid, as had been found by Hill, Long and Lupton (*Proc. Roy. Soc.*, 1924, B, **96**, 455) and Ronzoni and Laurence (*J. Biol. Chem.*, 1927, **74**, 363). 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, per cent. *	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, per cent. *	97·7	97·6	97·6	102·3	98·4	103·2	100·0	100·0

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, per cent.	80·4	82·3	80·3	78·9	80·3	82·6	76·3
Sulphuric acid method, found, per cent.	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

* Using factor (see p. 46.)

yield of 80 per cent. 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 per cent.

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 (*ibid.*) 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 per cent. 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 c.c. with 20 per cent. sulphuric acid; 20 c.c. of this solution were taken at intervals of 1½ 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, per cent.	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

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

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 c.c. of 17·5 per cent. 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 per cent. 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 per cent., i.e. 1 c.c. $N/10$ 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 reaction 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 (*J. Biol. Chem.*, 1917, **32**, 455) 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 (*J. Biol. Chem.*, 1919, **38**, 81) 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 per cent. alcohol added. The solution is kept overnight, filtered and the precipitate washed with 98 per cent. 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 per cent.	Method of precipitation	Pyruvic acid recovered, per cent.
4·61	2 hæmoglobin	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 per cent. 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 per cent., about 2 to 5 c.c. 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 c.c. 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 (*J. Biol. Chem.*, 1908, **5**, 211) 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 c.c. 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 (c.c.)	Control (c.c.)	Amount recovered using the empirical factor	Error
Sheep's blood, 5 c.c. ...	1·51	5·5 (0·005 *N*)	0·10?	1·49	−1·3
	3·02	11·3 (0·05 *N*)	0·10?	3·08	+2·0
	6·04	11·15 (0·01 *N*)	0·10?	6·08	+0·7
Yeast culture solution, 5 c.c. ...	1·51	5·65 (0·005 *N*)	0·25	1·49	−1·3
	3·02	11·12 (0·05 *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 c.c. ...	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 c.c. 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 per cent.

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.

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