XCI. THE DETERMINATION OF PYRUVIC ACID.

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In a previous paper [Case and Cook, 1931] reference was made to the need for an accurate quantitative method for the estimation of pyruvic acid in muscle and muscle-filtrates. The test used throughout the above work was the nitroprusside test of Simon and Piaux [1924]. This is sensitive and specific, the colour produced being easily distinguished from that given by any compound other than pyruvic acid. It is therefore extremely useful for the detection and approximate estimation of the latter. However, attempts which have since been made to develop a strictly quantitative colorimetric technique on these lines have proved fruitless owing to the fugacity of the colour and the fact that other substances present are liable to modify its tint.

It was thought that the phloroglucinol-HCl test of Posternak [1927] might be exploited, but although this is quite sensitive, the precise shade of colour is again interfered with by other substances to such an extent as to render it unsuitable for colorimetric purposes; moreover its specificity is not all that could be desired.

Methods based upon bisulphite-binding capacity [*e.g.* Cook, 1930] are not satisfactory, since pyruvic acid is far from being the only substance likely to occur in muscle which possesses this power. The same criticism applies to Wieland's [1924] method, depending upon iodoform formation.

Procedures involving reduction of the pyruvic acid to lactic acid and estimation of the latter compound [*e.g.* Lieben, 1923; Krishna and Sreenivasaya, 1928] are practically useless for muscle-filtrates on account of the large amounts of lactic acid already present.

Methods depending upon precipitation of pyruvic acid with phenylhydrazine and subsequent estimation of the unchanged hydrazine [MacLean, 1913] are clearly of no value when, as is the case in muscle, other hydrazone-forming substances are present also. Separation of the compounds formed with substituted hydrazines has hitherto been stated to be impracticable, since the solubility of the pyruvic acid derivatives is never sufficiently low to render possible anything approaching a quantitative precipitation. This obstacle has however been overcome in the present work, and a technique for the estimation

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of pyruvic acid in muscle-filtrates has been developed which depends upon the properties of the 2 : 4-dinitrophenylhydrazone of this substance.

Principle.

Ultimately this is similar to that of the method of Barrenscheen and Dreguss [1931] for the estimation of methylglyoxal; *viz.* the depth of colour produced by dissolving the 2 : 4-dinitrophenylhydrazone in alcoholic potassium hydroxide is determined colorimetrically against a standard. The difficulty raised by the appreciable solubility of this hydrazone in water is surmounted by extraction with ethyl acetate, followed by removal of the pyruvic acid derivative from the organic solvent by means of aqueous sodium carbonate. The corresponding hydrazones from a large number of other substances which may be present, *e.g.* methylglyoxal, acetaldehyde, *etc.* are not soluble in aqueous sodium carbonate.

Experimental details.

Deproteinisation of the tissue or tissue extract is carried out with trichloroacetic acid, which should be present in a final concentration of 2–5 %. 20 cc. of the clear filtrate are conveniently taken for the estimation, though if this quantity be not available, less can be used. (If more than 5 mg. of pyruvic acid are present in 20 cc. of filtrate it is advisable to dilute the latter accordingly and employ 20 cc. of the diluted solution.) 5 cc. of 1 % 2 : 4-dinitrophenylhydrazine (dissolved in 2 *N* HCl) are added and the mixture is kept for 2 hours at room temperature.

A visible precipitate may or may not appear, according to the amount of hydrazone-forming substances present. The whole is now shaken with 20 cc. of ethyl acetate in a glass-stoppered 50 cc. separating funnel. After separation, the aqueous layer, which is nearly colourless, is extracted with a further 10 cc. of ethyl acetate; as soon as the aqueous layer is colourless, as usually is the case after two extractions, it is discarded. The united ethyl acetate extracts now contain all the unchanged 2 : 4-dinitrophenylhydrazine together with the hydrazones which have been formed. The liquid is also acid, owing to the extraction of a certain amount of hydrochloric and trichloroacetic acids; these must be neutralised by shaking with solid calcium carbonate (Notes 2, 3). The solution is decanted into a glass evaporating basin, washing the calcium carbonate with further ethyl acetate until it is colourless. The washings are added to the main bulk of the fluid. The substances in solution must now be transferred to toluene (Note 4). This is accomplished by evaporating the contents of the dish on a water-bath to 1–2 cc. and then, after removal from the bath, adding about 20 cc. of toluene. The slightly cloudy yellow solution is again transferred to the separating funnel and is thoroughly shaken with 5 cc. of cold 25 % sodium carbonate solution (Note 5). If pyruvic acid was originally present its hydrazone dissolves in the aqueous layer, colouring it brown. This extraction is repeated with fresh Na_2CO_3 solution until the latter remains colourless; two or three repetitions usually suffice. The united sodium

carbonate layers are now acidified by adding concentrated hydrochloric acid drop by drop. The 2 : 4-dinitrophenylhydrazone of pyruvic acid is precipitated and a lemon-yellow suspension results. This is extracted in a separating funnel with successive 10 cc. portions of ethyl acetate until the aqueous layer is colourless (Note 6). It is not usually necessary to employ more than 20 cc. of ethyl acetate to achieve this. The ethyl acetate solution now contains all the pyruvic acid hydrazone which was present, and is evaporated to dryness in a glass basin on a water-bath. The yellow residue is dissolved when cool in 5 % alcoholic potassium hydroxide (Note 7), giving a deep red solution which is made up in a graduated vessel with further alcoholic potash to a volume such that the colour is suitable for reading in a colorimeter. In practice it is found that the total volume at this stage should be about 50 cc. for every mg. of pyruvic acid that was originally present in the sample taken.

For the purpose of the standard, a pure preparation of pyruvic acid 2 : 4-dinitrophenylhydrazone is made, and a stock solution of this is kept in ethyl acetate, of such a strength that 1 cc. is equivalent to 0·1 mg. pyruvic acid. The appropriate volume is taken, evaporated to dryness, and dissolved in alcoholic potash exactly as described above. As in all colorimetric work, the standards should be chosen so that the depth of colour obtained is as close as possible to that in the unknown when both are made up to the same volume. Preliminary tests show, however, that in the neighbourhood of the concentrations stated proportionality exists between the depth of colour and the amounts of hydrazone present.

Notes. 1. If sulphite or bisulphite is present, the acid filtrate should be boiled until no more SO_2 is given off. Sulphite interferes with the precipitation to some extent.

2. If this neutralisation be omitted, the subsequent heating of the solution leads to the formation of small quantities of compounds other than the pyruvic acid derivative which are extracted by the sodium carbonate, colouring it brown, and which are afterwards precipitated upon acidification, giving a reddish colour in the final treatment with alcoholic potash. When the neutralisation is properly carried out, the sodium carbonate always remains colourless in blank determinations, *i.e.* when no pyruvic acid is present.

3. It was at first the practice to neutralise the acid solution before extraction with ethyl acetate, but if this be done it is found that extraction has to be repeated a much larger number of times before the aqueous layer is colourless.

4. The solubility of the pyruvic acid derivative in ethyl acetate is so great that it is not completely removed by sodium carbonate solution. Moreover, shaking the ethyl acetate solution with sodium carbonate seems to lead to a small amount of saponification, with the result that separation into layers is imperfect. The substitution of toluene for ethyl acetate overcomes both of these difficulties, but it cannot be used for extraction at the outset because the hydrazine and hydrazones are not sufficiently soluble in it to render the process efficient.

5. The toluene extract and the sodium carbonate solution must both be at room temperature. Warm Na_2CO_3 reacts with other 2 : 4-dinitrophenylhydrazones besides that of pyruvic acid.

6. A good deal of care must be taken at this stage that the effervescence produced by the liberated CO_2 does not lead to loss by splashing.

7. A small quantity of sodium chloride and traces of trichloroacetate are always present at this stage, having been extracted by the ethyl acetate. It is advisable therefore first to add a drop or two of water to dissolve these substances, which may otherwise cause the final solution to be turbid. By reason of the presence of these admixtures, actual weighing of the hydrazone is impracticable.

Before giving experimental figures obtained by this method, it is worth while pointing out that pyruvic acid very readily undergoes changes of the nature of polymerisation or condensation, and that the purity of laboratory samples is therefore always of a low order. For the purposes of this work, Kahlbaum's pyruvic acid was twice distilled *in vacuo*, the fraction boiling at about 66°/15 mm. being collected. The colourless product was diluted with ice-cold water and accurately neutralised with sodium hydroxide, the temperature being kept below 5°. The solution was now concentrated in a vacuum desiccator; the glistening white plates of sodium pyruvate that crystallised out were powdered, washed thoroughly with boiling absolute alcohol, heated for some time at 100°, and stored *in vacuo* over sulphuric acid. In the determinations reported below, the pyruvic acid was added in the form of equivalent amounts of this sodium salt in aqueous solution of known concentration.

Table I sets forth the results of determinations made by this method when quantities of pyruvic acid varying between 0·1 mg. and 10·0 mg. were taken. It also shows that the presence of trichloroacetic acid does not interfere.

Table I.

Pyruvic acid dissolved in	Pyruvic acid taken (mg.)	Pyruvic acid found (mg.)	Percentage error
20 cc. water	0·10	0·094	−6·0
,,	0·15	0·145	−3·3
,,	0·20	0·200	0·0
,,	0·50	0·498	−0·4
,,	1·00	1·018	+1·8
,,	2·00	1·980	−1·0
,,	5·00	4·900	−2·0
,,	10·00	9·130	−8·7
20 cc. 5 % trichloroacetic acid	0·10	0·096	−4·0
,, ,,	0·15	0·147	−2·0
,, ,,	0·20	0·204	+2·0
,, ,,	0·50	0·490	−2·0
,, ,,	1·00	0·980	−2·0
,, ,,	2·00	2·000	0·0
,, ,,	5·00	4·880	−2·4
,, ,,	10·00	9·300	−7·0

It is to be seen from this table that under the conditions described the method works most satisfactorily for quantities of pyruvic acid between 0·2 mg. and 5·0 mg., which are estimable with a possible error not exceeding ± 2 %. Since a concentration of 0·2 mg. in 20 cc. represents a dilution of 1 part in 100,000, considerable sensitivity as well as accuracy can be claimed.

In Table II it is shown that the estimation is not interfered with by the presence of comparatively large amounts of a variety of other metabolites which may possibly be present in preparations from muscle. The pyruvic acid and other substances are dissolved throughout in 20 cc. of 5 % trichloroacetic acid.

The method has thus a high degree of specificity. Most of the substances in Table II form hydrazones with 2 : 4-dinitrophenylhydrazine, many of which give a red colour with alcoholic potash; but, of them all, pyruvic acid is

Table II.

Pyruvic acid taken (mg.)	Additions other than pyruvic acid	Pyruvic acid found (mg.)	Percentage error
0·20	50 mg. glucose	0·197	−1·5
5·00	,, ,,	5·050	+1·0
0·20	50 mg. fructose	0·194	−3·0
5·00	,, ,,	4·890	−2·2
0·20	50 mg. glycogen	0·200	0·0
5·00	,, ,,	5·044	+0·9
0·20	50 mg. starch	0·198	−1·0
5·00	,, ,,	4·900	−2·0
0·20	50 mg. methylglyoxal	0·202	+1·0
5·00	,, ,,	5·080	+1·6
0·20	50 mg. dihydroxyacetone	0·196	−2·0
5·00	,, ,,	5·065	+1·3
0·20	50 mg. glyceraldehyde	0·200	0·0
5·00	,, ,,	4·920	−1·6
0·20	50 mg. sodium hexosediphosphate	0·198	−1·0
5·00	,, ,,	5·050	+1·0
0·20	50 mg. sodium hexosemonophosphate	0·205	+2·5
5·00	,, ,,	5·092	+1·8
0·20	50 mg. acetaldehyde	0·200	0·0
5·00	,, ,,	4·950	−1·0
0·20	50 mg. formaldehyde	0·194	−3·0
5·00	,, ,,	4·900	−2·0
0·20	50 mg. acetone	0·203	+1·5
5·00	,, ,,	5·000	0·0

the only one whose hydrazone is extracted by sodium carbonate. It is probable that this property is associated with the presence in the molecule of the —COOH group, in addition to the ketonic group which is responsible for the formation of the hydrazone. For this reason other compounds containing groups of both these types would be expected to interfere. To take two such examples, glycuronic acid and laevulic acid have been tested. Both of these substances form hydrazones with 2 : 4-dinitrophenylhydrazine which are yellow, and which are soluble in aqueous sodium carbonate; both hydrazones yield a red colour with alcoholic potash. By this method, therefore, such compounds would be estimated as pyruvic acid. But in working with muscle, at all events, it is practically certain that the results obtained are to be ascribed in reality to pyruvic acid, and that no interfering substances of the type just discussed are present, for the following reasons.

(*a*) The results given by the method described invariably run closely parallel with observations made by means of the nitroprusside test; this is exemplified in another communication [Case, 1932].

(*b*) If the product obtained in the final stages of the procedure be further purified and examined, no hydrazone can be isolated from it other than one melting at 214°, and thus corresponding to pyruvic acid [Allen, 1930].

SUMMARY.

A method is described for the determination of pyruvic acid, with special application to muscle-filtrates. One part in 100,000 of pyruvic acid can be estimated, in absolute amounts at least as low as 0·2 mg. The specificity is discussed and shown to be of a high order.

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