XXX. STUDIES ON THE METABOLISM OF PYRUVIC ACID IN NORMAL AND VITAMIN B_1-DEFICIENT STATES

I. A RAPID, SPECIFIC AND SENSITIVE METHOD FOR THE ESTIMATION OF BLOOD PYRUVATE

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SINCE the appearance of the Meyerhof-Embden scheme pyruvic acid is known to be a normal intermediate metabolite of the carbohydrate breakdown of mammalian tissues, of yeast fermentation and of the respiration of higher plants. It is involved in the metabolism of embryos, bacteria and tumour tissues, and in the action of hormones and vitamins. It also forms a link between the metabolism of carbohydrate and amino-acids. For the study of pyruvate metabolism in man with relation to vitamin B_1-deficiency, however, a really suitable method has not hitherto been available.

No less than fifty descriptions of methods or modifications of previous methods for the identification and quantitative estimation of pyruvic acid have been reviewed by Wendel* [1932]. Since then numerous other methods have been described; there is the $NaHSO_3$-binding method of Clift & Cook [1932], the 2:4-dinitrophenylhydrazone method of Case* [1932], the carboxylase method for determination in blood [Westerkamp, 1933], the modification of the Neuberg-Case method [Peters & Thompson, 1934], the semicarbazide-HCl precipitation method and its modification [Hahn *et al.* 1934; 1936], the α-methylindole-HCl colour reaction for its estimation in blood [Dische & Robbins, 1934], the ceric sulphate method [Fromageot & Desnuelle, 1935], the red colour reaction of the 2:4-dinitrophenylhydrazone of pyruvic acid in alkaline solution [Jowett & Quastel, 1937], and finally the microchemical adaptation of the method of Clift & Cook for use with minute quantities of blood by de Jong & Picard* [1937].

Of all the methods mentioned above only a few are applicable to quantitative blood analysis because of the sensitivity required. Pyruvic acid is present in normal blood in extremely small amounts: below 0·6 mg./100 ml. The carboxylase method, apart from its questionable specificity, is too complicated for ordinary purposes. The ceric sulphate method is simple and rapid for estimating small quantities; but the interference of lactic acid prevents it from being adequate for analysis of biological fluids. Unfortunately neither of the two micro-methods described is really specific. The α-methylindole-HCl reaction of Dische & Robbins has been found by de Jong & Picard to be non-specific; the intensity of the colour developed varying with the concentration of the reagents and temperature. The author agrees with Peters & Thompson [1934] in finding that heating with alkali to remove substances other than pyruvic acid as described by Clift & Cook is not satisfactory when tissue or blood extracts are used. Many experiments of this type were done on beri-beri blood and the value never

* Criticisms of some of the previous methods are to be found in references marked with an asterisk.

agreed with that obtained by the elaborate hydrazone method: hence the micromethod described by de Jong & Picard may, like the estimation of bisulphite-binding substances, work well with pigeons' blood, but is not applicable to clinical studies. This leaves only the hydrazone method. It is specific, but suffers from the following disadvantages. First, for clinical purposes the procedure is complicated; secondly, the length of time required often renders it of no value to the physician, either for diagnosis or treatment, since acute beriberi patients usually die a few hours after admission; and thirdly the venous puncture necessary in order to obtain enough blood (at least 1·2 ml.) for every test may be an important obstacle to research studies on the changes in human or animal blood pyruvate. It is therefore necessary to have a method which is simple, specific, rapid and needs only small quantities of blood.

The difficulty in the quantitative estimation of pyruvic acid is the impossibility of separating it from other aldehyde or ketone derivatives present. In colorimetric reactions these compounds form colours which are liable to modify the tint. It is possible to develop a colorimetric determination of pyruvic acid in small quantities by the selection of optimal specific conditions in which the colour of the pyruvic acid hydrazone is maximal and permanent, and by the use of a light filter.

The principle is as follows: pyruvic acid is first converted into its 2:4-dinitrophenylhydrazone. This is extracted with ethyl acetate from the aqueous solution. The hydrazone of pyruvic acid is separated from the excess of hydrazine added and the other hydrazones of aldehyde or ketone derivatives by extraction with Na_2CO_3. Traces of the hydrazine or hydrazone carried over by the ethyl acetate dissolved in the aqueous Na_2CO_3 are removed with a fresh lot of ethyl acetate. The stable red colour developed by adding NaOH to the Na_2CO_3 extract of the 2:4-dinitrophenylhydrazone of pyruvic acid is determined colorimetrically, using a photoelectric colorimeter.[1] The interference of the yellow colour is eliminated by the use of a light filter.

Experimental details

Reagents required:

5 % and 10 % trichloroacetic acid,
0·1 % 2:4-dinitrophenylhydrazine in 2*N* HCl,
Ethyl acetate, pure,
N NaOH,
10 % Na_2CO_3.

2 or 3 drops of freshly shed blood are dropped directly into an accurately weighed 10 ml. centrifuge tube containing 1·0 ml. 10 % trichloroacetic acid and previously cooled to below 0°. After mixing well, it is weighed again to ±0·1 mg. or it may be convenient to use the 0·2 ml. special blood pipette described by Harrison [1937] for micro-sugar analysis. The precipitated protein is centrifuged down 10 min. after the blood is taken. The clear supernatant liquid is transferred quantitatively to an ordinary test tube (tube 1). The protein precipitate is mixed with 1 ml. 5 % trichloroacetic acid, and after being allowed to stand for 2 min. is centrifuged again and the supernatant liquid is transferred to tube 1. To the combined trichloroacetic extracts is added 1 ml. 2:4-dinitrophenylhydrazine solution. The mixture is allowed to stand at room temp. for not less than 10 min. It is then extracted with 2 ml. ethyl acetate and well mixed. When the two layers have separated out, the lower acid layer is quantitatively

[1] This is made by the Unicam Instrument Company, Cambridge, on the lines of the Evelyn Colorimeter [Evelyn, 1936].

removed with a fine-tipped dropper to tube 2, and is there extracted again with 1·0 ml. ethyl acetate. The upper ethyl acetate layer is carefully transferred to tube 1, and the lower layer is extracted for a third time with 1·0 ml. ethyl acetate. The clear aqueous layer is now discarded and the ethyl acetate added to tube 1. The combined ethyl acetate extract contains all the unchanged hydrazine added, and the hydrazones formed. From a burette exactly 2·0 ml. Na_2CO_3 are added to tube 2, rinsed well and the whole poured into tube 1. This is then mixed well with the aid of the same dropper and allowed to extract for at least 3 min. When the separation is completed the Na_2CO_3 layer is returned to tube 2. This extraction with Na_2CO_3 is repeated twice again with 2 ml. portions of Na_2CO_3 each time. The combined Na_2CO_3 solution in tube 2 is extracted for the last time with 1·0 ml. of ethyl acetate. The clear Na_2CO_3 extract, which may or may not be coloured yellow, depending on the quantity of the hydrazone present, is quantitatively transferred to a clean tube 3 to which 4·0 ml. *N* NaOH are added. A stable red colour develops which is determined after 10 min., using a photoelectric colorimeter with Wratten No. 62 light filter. The exact amount of pyruvic acid present in the given sample is read off from the standard curve.[1]

The standard curve is constructed as follows. A pure preparation of the 2:4-dinitrophenylhydrazone dissolved in ethyl acetate solution can be used, but a pure pyruvate solution is preferred. The pure pyruvic acid solution is prepared by three redistillations *in vacuo*, the fraction boiling at 55–60°/10 mm. Hg being collected. The clear liquid crystallizes out on standing in a freezing mixture at −4°. The crystals, carefully weighed in a weighing bottle, are dissolved in 50 vol. of ice-cold freshly distilled water. After neutralization with NaOH,

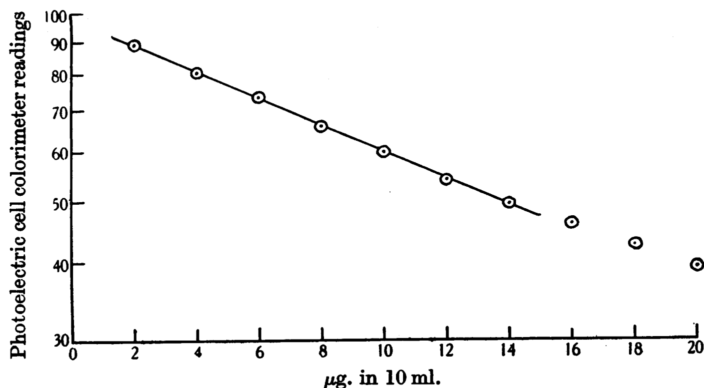

Fig. 1. Standard curve.

care being taken not to let the temperature rise, the pyruvate solution is diluted to 1%. The exact concentration of the solution is checked by estimating its bisulphite-binding power. The standard pyruvate solution thus prepared is further diluted to a concentration of 5 µg./ml. Duplicate series of tubes, containing pyruvic acid from 2 to 20 µg., are set up, and estimations carried out in the manner described above for blood analysis. The red colour developed is stable from the first min. for more than 90 min. in solutions of pure pyruvate

[1] It is always necessary to prepare a blank of trichloroacetic acid and hydrazine, extracted in the usual manner, to be used as a control solution for setting the zero of the photoelectric colorimeter. If the experiment is well carried out the blank is practically colourless every time. After each reading the galvanometer should return to the original mark.

hydrazone. The deflexions of the galvanometer readings when plotted against concentrations of pyruvic acid on arithlog paper give a straight line (Fig. 1).

The procedure just described requires a few further comments. As the method is extremely sensitive, it is essential to take every precaution to prevent loss of traces of the extract containing the pyruvic acid 2:4-dinitrophenylhydrazone by avoiding the use of a separating funnel or of numerous test tubes. We differ from previous authors, who worked on a much larger scale and in presence of a great excess of 2:4-dinitrophenylhydrazine, in finding that the Na_2CO_3 under the conditions described completely removes the pyruvic acid derivative as shown by the recovery data. Moreover, mixing gently with the aid of the dropper instead of shaking in a separating funnel gives rise to no foaming and hence no interference with the perfect separation of the two layers. Na_2CO_3 is to be preferred to Na_2HPO_4 as the latter gives a much inferior colour at the last stage. The combined techniques of extracting with ethyl acetate to remove impurities at an alkaline reaction, using a comparatively small but sufficient amount of hydrazine and developing the red colour in the aqueous Na_2CO_3 extract of the hydrazone enable us to eliminate the subsequent neutralizations and re-extractions with ethyl acetate, and the drying at the last stage, which are so laborious and liable to introduce error. 2:4-Dinitrophenylhydrazones of other keto-acids also give a red colour, but in the concentrations present in human biological fluids, whether normal or in ketosis, these substances do not interfere. Under the experimental conditions described, if they are present in larger amounts, their colour fades into yellow on standing at room temp. for 10 min. after the addition of NaOH. This reaction as used by Jowett & Quastel is not applicable to the estimation of pyruvic acid changes for studies on the physiology or pathology of animals, because the changes in blood pyruvic acid are so small [Thompson & Johnson, 1935; Platt & Lu, 1935; 1936] and the method is not specific enough.

A comparison of pyruvate values was made between the present method and the modified Neuberg-Case hydrazone method [Peters & Thompson, 1934]. The results are shown in Table I.

Table I

	mg. per 100 g.			
Specimen used	Macro-method (Neuberg-Case)	New micro-method	Difference	Micro-method % of macro-method
Blood	0·765	0·770	0·005	101
Milk	0·584	0·570	0·014	98
Cerebro-spinal fluid	0·940	0·951	0·011	101

From these figures it is obvious that the methods are in good agreement.

Sensitivity and specificity of the method

The method estimates 2 μg. in 10 ml., a dilution of 1 : 5,000,000 with an error of $\pm 1{\cdot}5\%$. It is 1000 times more sensitive than the reaction of Simon & Piaux [1924] and 50 times more sensitive than the method of Case [1932]. Since the same principle of isolating the hydrazone of keto-acids by carbonate extraction is used in the new method its specificity is at least as high as that of the Neuberg-Case method. Indeed the use of the light filter has rendered it much more specific when small amounts of other keto-acids have been added. How far this is important for practical purposes in studies of blood in diseased conditions is not yet determined.

For many years acetoacetic acid has been known to increase in the blood in ketosis. Recently Krebs [1938] has found α-ketoglutaric acid to be present in human blood and urine, while oxaloacetic acid has been found by numerous authors to play a part in tissue metabolism. It is possible that laevulic and glucuronic acids may also prove to be of physiological importance. It may therefore be necessary to determine the total amount of keto-acids present. This may be done by substituting 15% Na_2CO_3 for 10%, by using $3N$ instead of N NaOH, and by taking the reading 2 min. after mixing. Under these conditions all the keto-acids give maximum colour.

On the other hand, under the conditions described in this paper, where pyruvic acid alone is to be estimated, the relative interference is as follows:

	Deflexion of the photo-cell colorimeter reading equivalent to 1 μg. pyruvic acid
Acetoacetic acid	59 μg.
Laevulic acid	15 ,,
α-Ketoglutaric acid	7·5 ,,

Oxaloacetic acid, if present, is of course always estimated as pyruvic acid, being unstable in acid solution. The total keto-acids could be estimated after a pyruvic acid determination by adding concentrated Na_2CO_3 and NaOH to make the final proportions as indicated above, and then measuring the colour again in the colorimeter. In this case, however, an appropriate calibration curve would have to be worked out.

The present method possesses yet further advantages over the standard Neuberg-Case method, for whereas the application of the latter to urine often leads to yellowish brown colours and gives purplish ones with tissue extracts, none of these inconveniences are met with in the present method.

Application of the method to the analysis of cerebro-spinal fluid, milk, urine and muscle involves no change except in the preparation of the pyruvic acid extract. Deproteinization of cerebro-spinal fluid and urine is carried out in the same manner as with blood. For tissue 8 parts of 5% CCl_3COOH are used for every g. In the case of milk, 1 ml. of 75% $(NH_4)_2SO_4$ is used in place of 1 ml. 10% and 5% trichloroacetic acid. Tables II and III illustrate the recoveries of added pyruvate in blood and milk.

Table II. *Recovery of pyruvic acid from human blood*

Pyruvic acid found in 100 ml. blood mg.	Pyruvic acid added per 100 ml. mg.	Pyruvic acid recovery	
		Found mg./100 ml.	Calc. mg./100 ml.
0·56 (normal)	2·0	2·52	2·56
0·98 (subacute beri-beri)	1·0	1·93	1·98
2·35 (acute beri-beri)	0·5	2·82	2·85

Table III. *Recovery of pyruvate from milk*

Pyruvic acid found in 100 ml. milk mg.	Pyruvic acid added per 100 ml. mg.	Pyruvic acid recovery	
		Found mg./100 ml.	Calc. mg./100 ml.
0·24	0·75	0·98	0·99
0·34	1·00	1·34	1·34
0·53	0·50	1·06	1·03

Recoveries from tissue extracts are of the same order.

SUMMARY

The colour of the 2:4-dinitrophenylhydrazone of pyruvic acid may be used for the rapid, specific and sensitive micro-estimation of this substance in biological fluids if new extraction procedures for removing interfering substances are adopted, and if a light filter is used in the photoelectric cell colorimeter to increase accuracy by eliminating accompanying traces of other tints.

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