Pyruvate

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The principle of the estimation of substrates of the glycolytic enzymes by means of the spectrophotometric measurement of the pyridine nucleotides was developed by *O. Warburg* and his school. A method for the determination of pyruvate in plasma was described by *Kubowitz* and *Ott*[1] in 1943. Since then the enzymatic estimation of pyruvate with lactic dehydrogenase has replaced other methods (*e.g.* the dinitrophenylhydrazone methods[2,3]), because of its simplicity and specificity.

Principle

Lactic dehydrogenase (LDH) catalyses the reduction of pyruvate with reduced diphosphopyridine nucleotide (DPNH):

$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{DPN}^+$$

The equilibrium of the reaction is very much in favour of lactate formation. The value for the equilibrium constant[4] measured at 25°C is 4×10^{11} [l./mole] at pH 0; 4×10^{4} [l./mole] at pH 7.0 and 1×10^{4} [l./mole] at pH 7.6. Provided there is a sufficient excess of DPNH the reaction proceeds rapidly to completion and pyruvate is quantitatively converted to lactate. The decrease of optical density due to the oxidation of DPNH is measured.

Reagents *)

1. Potassium carbonate, K_2CO_3, A. R.
2. Methyl orange
3. Perchloric acid, A. R., sp. gr. 1.67; *ca.* 70% (w/w)
4. Triethanolamine hydrochloride
5. Sodium hydroxide, A. R., 2 N
6. Ethylene-diamine-tetra-acetic acid,
   disodium salt, EDTA-$Na_2H_2 \cdot 2\ H_2O$
7. Sodium hydrogen carbonate, 1% (w/v)
8. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
9. Lactic dehydrogenase, LDH
   crystalline, from skeletal muscle, suspension in 2.1 M ammonium sulphate solution. Commercial preparation, see p. 986.

Purity of the enzyme preparation

The LDH preparation should have a specific activity of at least 15000 units/mg. according to *Bücher*[5], equivalent to *ca.* 270 units/mg. according to *Racker*[6]. To ensure complete specificity of

*) Complete reagent kits are available commercially, see p. 1036.

1) *F. Kubowitz* and *P. Ott*, Biochem. Z. *314*, 94 [1943].
2) *T. E. Friedemann* and *G. E. Haugen*, J. biol. Chemistry *147*, 415 [1943].
3) *S. Markees* and *F. W. Meyer*, Schweiz. med. Wschr. *1949*, 931; *S. Markees*, *O. Käser* and *R. Lang*, Schweiz. med. Wschr. *1950*, 1079; *S. Markees*, Helv. physiol. pharmakol. Acta *9*, C. 30 [1951].
4) *H. J. Hohorst*, *F. Kreutz* and *Th. Bücher*, Biochem. Z. *332*, 18 [1959].
5) *G. Beisenherz*, *H. J. Boltze*, *Th. Bücher*, *R. Czok*, *K. H. Garbade*, *E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953].
6) *J. Cooper*, *P. A. Srere*, *M. Tabachnik* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].

the determination the LDH preparation should not contain more than 0.01% pyruvic kinase, glycerophosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and malic dehydrogenase (relative to the specific activity of the LDH).

Preparation of Solutions (for *ca.* 20 determinations)

Sterilize all reagent bottles before use in order to prevent bacterial contamination.

I. Potassium carbonate (*ca.* 5 M):
Dissolve *ca.* 69 g. K_2CO_3 in doubly distilled water and make up to 100 ml.

II. Methyl orange indicator (0.05% w/v):
Dissolve 50 mg. methyl orange in doubly distilled water and make up to 100 ml.

III. Perchloric acid (*ca.* 6% w/v):
Dilute 7.8 ml. 70% $HClO_4$ to 150 ml. with doubly distilled water.

IV. Triethanolamine buffer (0.4 M; pH 7.6):
Dissolve 18.6 g. triethanolamine hydrochloride in 200 ml. doubly distilled water, add 18 ml. 2 N NaOH and 3.7 g. EDTA-$Na_2H_2 \cdot 2\ H_2O$ and dilute to 250 ml. with doubly distilled water.

V. Reduced diphosphopyridine nucleotide (*ca.* 5×10^{-3} M β-DPNH):
Dissolve 7 mg. DPNH-Na_2 in 1.5 ml. doubly distilled water.

VI. Lactic dehydrogenase LDH (*ca.* 10 mg. protein/ml.):
Dilute the enzyme suspension with 2.1 M ammonium sulphate.

Stability of the solutions

All solutions should be stored in a refrigerator between 0 and 4° C. The DPNH solution must be freshly prepared each week. The other solutions are stable indefinitely as long as no bacterial contamination occurs.

Procedure

Experimental material

Blood should be taken from veins without stasis and the sample should be immediately deproteinized. Owing to the glycolytic activity of red cells, estimations on serum are not valid. When plasma is to be examined it should be separated as quickly as possible from the cells in the cold. For estimation in tissues, the sample should be frozen within a fraction of a second and should not be thawed until ready for deproteinization[4].

Deproteinization

Preliminary remarks: The deproteinization is carried out with perchloric acid (solution III). The amount is governed by the water content of the sample. Sufficient perchloric acid is added so that the ratio of the final total liquid volume of the sample to its original weight is 4 : 1. The water content of blood is assumed to be 80% and of tissues (liver, kidney, muscle, heart) 75%. Therefore:

to 2 g. of blood add 6.4 ml. perchloric acid (solution III)
to 2 ml. of blood add 6.3 ml. perchloric acid (solution III)
to 2 g. of tissue add 6.5 ml. perchloric acid (solution III).

A variation of ±10% from the assumed value for the water content causes an error of ±2.5% in the analytical results. It is usually easier to weigh blood samples. If it is preferred to measure volumes of blood, then it should be noted that the graduations of commercial syringes may have considerable errors. For conversion the specific gravity of blood is 1.06 g./ml.

Method: Prepare a graduated centrifuge tube containing a glass rod (thick-walled tubing, blown to a bulb at the bottom) with

4 ml. perchloric acid (solution III).

Weigh the tube and add

2 ml. sample

(blood flowing direct from a cannula, frozen blood or frozen powdered tissue, until the volume has increased by 2 ml.), mix and reweigh. From the increase in weight calculate the total volume of perchloric acid required (for amounts, see under "Preliminary remarks"). To the original 4 ml. add sufficient

perchloric acid (solution III)

to give this volume. Mix the suspension thoroughly. After 5 min. (with frozen tissue samples, calculated from when they are completely thawed), centrifuge (at least 3000 g, but preferably at higher speed) for 5 min. Separate protein particles adhering to the surface of the liquid by shaking and re-centrifuging. Pipette

4.00 ml. supernatant fluid
0.01 ml. indicator solution (II)

into a cooled 10 ml. vessel*). To neutralize**) add

about 0.1 ml. carbonate solution (I)

from a 0.2 ml. capillary pipette, while stirring vigorously with a magnetic stirrer. Wait until the CO_2 evolution has practically ceased, continue the titration until the end-point of the indicator is reached (pH *ca.* 3.5, salmon-pink). A total of about 0.14 ml. of carbonate solution is required. Allow the mixture to stand for 10 min. in ice water and then decant or pipette off the fluid from the precipitated potassium perchlorate. Analyse a portion of this supernatant fluid†).

Spectrophotometric measurements

Preliminary remarks: The ratio of assay volume to deproteinized solution taken is so arranged that no further calculations are necessary for measurements at different wavelengths. Measurements are made against a control cuvette, which has an extinction slightly higher than that of the sample solution minus the optical density due to DPNH. This results in the initial readings being on the most accurate range of the spectrophotometric scale, and also guarantees an excess of DPNH even if the zero of the scale is reached.

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*) The container (*e.g.* penicillin bottle) is cooled by placing it in the middle of a refrigerator ice tray from which one or two ice cubes have been removed.

**) Over-neutralization results in low values; in this respect pyruvate is more susceptible than the majority of other metabolites found in biological extracts.

†) The deproteinized solution can usually be stored for several days at 0° C, nevertheless it is recommended that the estimation be carried out immediately. Freezing or lyophilization results in a considerable lowering of the pyruvate values.

Method: Bring the solutions to room temperature and pipette in the given order into the cuvettes:

Wavelength: 340 mμ; light path: 1 cm.; final volume: 3.041 ml.

Experimental cuvette	*Control cuvette*
2.00 ml. deproteinized sample	2.00 ml. buffer (solution IV)
1.00 ml. buffer (solution IV)	0.03 ml. indicator solution (II)
0.04 ml. DPNH solution (V)	

Wavelength: 366 mμ; light path: 2 cm.; final volume: 3.952 ml.

Experimental cuvette	*Control cuvette*
2.50 ml. deproteinized sample	4.00 ml. buffer (solution IV)
1.40 ml. buffer (solution IV)	0.05 ml. indicator solution (II)
0.05 ml. DPNH solution (V)	

Wavelength: 334 mμ; light path: 1 cm.; final volume: 2.842 ml.

Experimental cuvette	*Control cuvette*
2.00 ml. deproteinized sample	2.80 ml. buffer (solution IV)
0.80 ml. buffer (solution IV)	0.03 ml. indicator solution (II)
0.04 ml. DPNH solution (V)	

Read the initial optical density E_1 (experimental against control cuvette) twice*) with an interval of 3 min. Pipette onto a small glass spatula

0.001 ml. or 0.002 ml. LDH suspension (VI)

(the former amount for measurements at 340, and the latter for 366 or 334 mμ) and stir thoroughly into the solution in the experimental cuvette. The final value E_2 is read at 3 and 6 min. after addition of the enzyme*).

Any changes during 3 min. in the optical densities E_1 and E_2 compared to the decrease in optical density can usually be disregarded. If E_2 shows a large change in 3 min. then this can be subtracted from the decrease in optical density. The decrease in optical density $\Delta E = E_1 - E_2$ (corrected where necessary) is used for the calculations.

If during the reaction the zero point of the spectrophotometer scale is reached, indicating that the amount of DPNH added is insufficient, the following method can be applied. On completion of the reaction pipette 0.05 ml. DPNH solution (V) into the experimental cuvette and read the new end value E_3. Prepare another cuvette containing 4 ml. triethanolamine buffer (solution IV) (for measurements at 340 or 334 mμ, use 3.0 or 2.8 ml. respectively). Measure the optical density E_4 against air, then pipette in a further 0.05 ml. DPNH solution (V) and measure optical density E_5 against air. Add the increase in optical density $E_5 - E_4 = \Delta E_2$ to the initial optical density E_1 of the experimental cuvette and from the sum $(E_1 + \Delta E_2)$ subtract the final value E_3. $E_1 + \Delta E_2 - E_3 = \Delta E$ is used for the calculations.

Calculations

The reaction proceeds stoichiometrically under the given conditions. The pyruvate content can be calculated in the usual way (see under "Calculation of Experimental Results" p. 37).

*) For the exact extrapolation to obtain the true value of ΔE, see p. 39.

Therefore:

(2) $$\frac{\Delta E \times V}{\varepsilon \times d} = \mu\text{moles pyruvate/assay mixture}$$

or

(3) $$\frac{\Delta E \times V}{\varepsilon \times d \times A} = \mu\text{moles pyruvate/g. tissue}$$

where

V = volume of the assay mixture [ml.]

A = portion of tissue [g.] = g. tissue $\times \frac{\text{ml. extract taken for assay}}{\text{total ml. extract}}$

ε = extinction coefficient of DPNH (for values, see below)

d = light path [cm.] of the cuvette

With the method described above, all further calculations are unnecessary because the ratio of [assay volume] to [volume of deproteinized sample in assay] is so arranged that:

(4) $$\frac{V}{A} \text{ is numerically equal to } \varepsilon \times d$$

Consequently, if the numerical value for V is substituted in equation (3) by $\varepsilon \times d \times A$, then

(5) ΔE is numerically equal to μmoles pyruvate/g. tissue.

To fulfil the requirements of equation (2) the following conditions are chosen:

at 366 mμ (ε = 3.3 cm.^{2}/μmole) the light path = 2 cm., the ratio V : A = 6.6
at 340 mμ (ε = 6.3 cm.^{2}/μmole) the light path = 1 cm., the ratio V : A = 6.3
at 334 mμ (ε = 5.9 cm.^{2}/μmole) the light path = 1 cm., the ratio V : A = 5.9

For example, for 366 mμ the ratio V:A = 6.6 is obtained by using the assay volume V = 3.952 ml. and by the content of A = 0.602 g. tissue in 2.5 ml. deproteinized sample (V:A = 3.952:0.602 $\approx$6.6).

Any method for the deproteinization of tissue can result in an error of $\pm 2\%$ in the analysis. Small fluctuations in the amount of potassium carbonate required for neutralization need not therefore be taken into account.

In cases where the pyruvate content of the tissue is low, it is recommended to carry out the measurements at 340 mμ or 334 mμ and to use cuvettes of greater light path. A decrease in the proportion of perchloric acid to tissue is not recommended.

Example

Normal blood: To 4 ml. of perchloric acid were added 2.16 g. blood. A further 2.9 ml. of perchloric acid were added in order to obtain the required proportion of 2 g. blood to 6.4 ml. perchloric acid ($2.16/2 \times 6.4 = 6.9$ ml.).

Measurements at 366 mμ against a blank cuvette:

before addition of LDH	0 min. $E_1 = 0.430$	ΔE
	3 min. $E_1 = 0.428$	
after addition of LDH	3 min. $E_2 = 0.322$	
	6 min. $E_2 = 0.320$	

$\Delta E = E_1 - E_2 = 0.430 - 0.322 = 0.108$ μmoles pyruvate/g. (ml.) blood.

Further Determinations

Other metabolites can be estimated in the same test solution by addition of specific enzymes before or after the pyruvate estimation. For example, dihydroxyacetone phosphate by addition of α-glycero-

phosphate dehydrogenase, and fructose-1,6-diphosphate by the addition of aldolase containing triosephosphate isomerase.

Sources of Error

1. The optical density of DPNH is slightly dependent on temperature at 366 mμ, but not at 340 or 334 mμ. Owing to the addition of the cold extract, the cuvette contents should be brought to room temperature before starting the measurements.

2. There occur in erythrocytes variable amounts of a compound which causes a slow decrease in the optical density of DPNH ("drift", refer to p. 39.) This effect is occasionally found with blood, rarely with tissue and never with plasma analyses. The described method of deproteinization and the assay conditions help to counteract this effect. It is more marked with smaller proportions of perchloric acid to tissue and also when the test is carried out in phosphate buffer. Subtraction of the 3 min. change in the initial or final optical density from the decrease in optical density is usually sufficient to correct for this effect. If the change in the final optical density is considerably higher than that due to "drift", then the presence of slow reacting keto acids is possibly indicated. Enzyme contamination is unlikely because of the activity and purity of the lactic dehydrogenase preparation.

3. Apart from the utilization of DPNH for the reduction of pyruvate, a decrease in optical density can also be caused by dilution of the assay solution during addition of the enzyme preparation. In the method described above the addition is so small that a correction is unnecessary. On adding larger volumes the ΔE should be corrected for the volume ratio before and after the enzyme addition.

Specificity

The specificity of muscle lactic dehydrogenase has been studied by *Meister*[7], *Holzer et al.*[8], *Franke et al.*[9] and *Friedmann et al.*[10]. β-Hydroxypyruvate and glyoxylate are reduced at a similar rate to pyruvate, while α-ketobutyrate and α-keto-n-valerate are reduced at considerably slower rates (see Fig. 1). The α-keto analogues of valine , leucine, isoleucine, phenylalanine, tyrosine, glutamic and aspartic acids are not attacked to any extent. Decomposition of oxaloacetate during the tissue extraction and carrying out of the assay, yields pyruvate. The rate of pyruvate formation from oxaloacetate in the assay solution is about 0.1 % per minute.

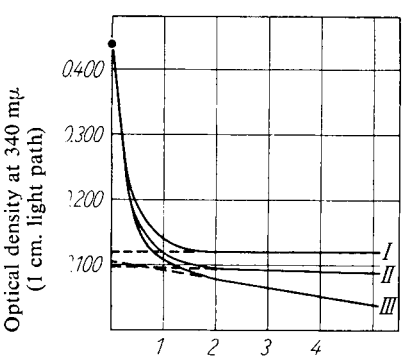

Fig. 1
The specificity of muscle dehydrogenase.
For experimental conditions, see text.
Curve I. 5×10^{-2} μmoles pyruvate/ml.
Curve II. as I., plus 2.5 μmoles α-keto-n-valerate/ml.
Curve III. as I., plus 5×10^{-2} μmoles α-ketobutyrate/ml.

7) *A. Meister*, J. biol. Chemistry *197*, 309 [1952].
8) *H. Holzer, W. Goedde* and *S. Schneider*, Biochem. Z. *327*, 245 [1955].
9) *W. Franke* and *W. Holz*, Hoppe-Seylers Z. physiol. Chem. *314*, 22 [1949].
10) *B. Friedmann, H. J. Nakada* and *S. Weinhouse*, Fed. Proc. *10*, 185 [1951].

If deproteinized, acid solutions from rat liver or human blood are heated for 6 minutes at 100°C a substance is liberated which reacts as pyruvate[11]. The nature of this substance is not yet known, Preliminary experiments on blood indicate that it is neither oxaloacetate nor phosphoenolpyruvate. An example of the changes in "extra pyruvate" in human blood after a meal is given in Table 1 (although no generalization can be made).

Table 1. Example of the effect of *Lamprecht* and *Latzko*[11] (see Text)

Min. after meal	Pyruvate after heating perchloric acid extract [% of unheated values]
30	100
60	125
120	110

[11] *W. Lamprecht* and *E. Latzko*, unpublished.