D-Lactate*)

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According to *Haugaard*[2] *E.coli B* oxidizes L- and D-lactate. However, an acetone extract of these bacteria in the presence of methylene blue oxidizes only D-lactate. The method[3] described here was specially developed for the determination of D-lactate in the culture media of micro-organisms, but it can be used without alteration for other experimental material.

Principle

Acetone extracts of *E.coli B* contain an enzyme system which oxidizes lactate to pyruvate and transfers the hydrogen to methylene blue:

(1) D-Lactate $\cdots\rightarrow$ pyruvate + (2 H)

(2) Methylene blue + (2 H) $\longrightarrow$ leuco-methylene blue

Leuco-methylene blue is spontaneously reoxidized by atmospheric oxygen to give methylene blue:

(3) Leuco-methylene blue + $^1/_2\,O_2$ $\longrightarrow$ methylene blue + H_2O

The series of reactions are irreversible and D-lactate is quantitatively oxidized. Each µmole D-lactate requires $^1/_2$ µmole oxygen (11.2 µl).

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Dipotassium hydrogen phosphate, K_2HPO_4
3. Potassium hydroxide
4. Methylene blue
5. Acetone powder of *E.coli B*

   E.coli B is grown on a synthetic culture medium containing DL-lactate as the only carbon source. The centrifuged cells are treated several times with acetone[3]. The dry powder is stable for at least two years at 4° C. The preparation must give virtually no reaction with L-lactate. Not every strain of *E.coli B* is suitable for the production of a usable enzyme preparation.

Preparation of Solutions

I. Phosphate buffer (0.2 M; pH 7.5):
Dissolve 4.08 g. KH_2PO_4 (anhydrous) and 29.58 g. K_2HPO_4 (anhydrous) in distilled water and make up to 1000 ml.

II. Potassium hydroxide (*ca.* 10% w/v):
Dissolve *ca.* 10 g. KOH in distilled water and make up to 100 ml.

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*) In order to avoid misunderstandings, note the following: D-Lactate refers to salts of laevorotatory lactic acid. *Labeyrie* et al.[1] have called attention to the fact that the expression, D(−)lactate, is a contradiction in terms, because the salts of D-(−)lactic acid are generally dextrorotatory.

1) *F. Labeyrie, P. P. Slonimski* and *L. Nastin*, Biochim. biophysica Acta *34*, 262 [1959].
2) *N. Haugaard*, Feder. Proc. *9*, 182 [1950].
3) *C. J. A. v. d. Hamer* and *R. W. Elias*, Biochim. biophysica Acta *29*, 556 [1958].

III. Methylene blue (*ca.* 2.5% w/v):
Dissolve *ca.* 2.5 g. methylene blue in distilled water and make up to 100 ml. Filter if necessary.

IV. Enzyme preparation from *E.coli B* (for *ca.* 10 determinations):
Grind 350 mg. acetone powder[3] with 10 ml. phosphate buffer (solution I) in a small mortar until homogeneous.

Stability of the solutions

Solutions II and III are stable indefinitely at room temperature. Solution I should be stored in a refrigerator in order to retard the growth of moulds. Preparation IV is stable for a few days at 4° C, but it then must be re-ground until homogeneous.

Procedure

Experimental material

Generally the culture media of micro-organisms do not require to be deproteinized. It is usually sufficient to centrifuge in the cold (15 min. at 3000 g).
Perchloric acid, zinc hydroxide or other reagents which do not inhibit the activity of the enzyme system may be used for deproteinization.

Manometric measurements

The determination is carried out with a Warburg apparatus (refer to p. 29). The reaction flasks must be fitted with a centre well for alkali *) and a side-arm for the sample. Two extra vessels, in addition to those for the samples, are required: one to correct for temperature and barometer changes ("thermobarometer") and one to measure any small O_2 uptake by the acetone powder ("O_2-blank"). Therefore for one sample, three flasks are necessary. An experimental protocol is given in Table 1:

Table 1. Contents of the manometer flasks

Compartment of flask	Solution	Thermo-barometer	O_2 blank	Sample
Centre well	potassium hydroxide (solution II)	0.1 ml.	0.1 ml.	0.1 ml.
Side-arm	sample	--	--	0.1 to 0.6 ml. **)
Main	enzyme preparation (solution IV)	—	1.0 ml.	1.0 ml.
	buffer (solution I)	1.0 ml.	--	—
	methylene blue (solution III)	—	0.3 ml.	0.3 ml.
	distilled water	dilute to standard volume of the flasks		

Connect the flasks to the manometers and place in a Warburg bath (37° C) with the manometer taps open. The temperature equilibration period is usually complete after 10—15 min. Close the manometer taps, note the level of the manometer fluid and mix the sample into the solution contained in the main compartment by tipping the reaction flask. Take readings of the manometers at 5 min. intervals until the pressure changes are small

*) Although no CO_2 is formed on oxidation of D-lactate, alkali is used in case other compounds present in the sample yield CO_2.
**) According to the size of the side-arm. The sample should contain at least 1 μmole D-lactate.

and equal those of the O_2-blank. After three further readings open the manometer taps, remove the flasks from the bath and quickly rinse them out, otherwise the enzyme suspension sticks to the walls.

The time taken for the determination depends on the D-lactate content of the sample and on the speed of shaking (mixing of the liquid with the gas).

Calculations

From the manometer readings the O_2 uptake for each flask is calculated[4], refer to p. 40. Preferably the results are set out in a table which, apart from a column for time of readings, should contain the following columns:

"Thermobarometer" (a): manometer readings

(b): (a) minus manometer reading before mixing sample into the main compartment

"O_2-blank" (c): manometer readings

(d): (c) minus manometer reading before mixing sample into the main compartment

(e): (d) minus (b)

(f): (e) multiplied by the flask constant (= O_2 uptake in µl.)

"Sample" (g): manometer readings

(h): (g) minus manometer reading before mixing sample into the main compartment

(i): (h) minus (b)

(j): (i) multiplied by the flask constant (= O_2 uptake in µl.)

(k): (j) minus (f) (= O_2 uptake in µl., corrected for the O_2 uptake of the acetone powder)

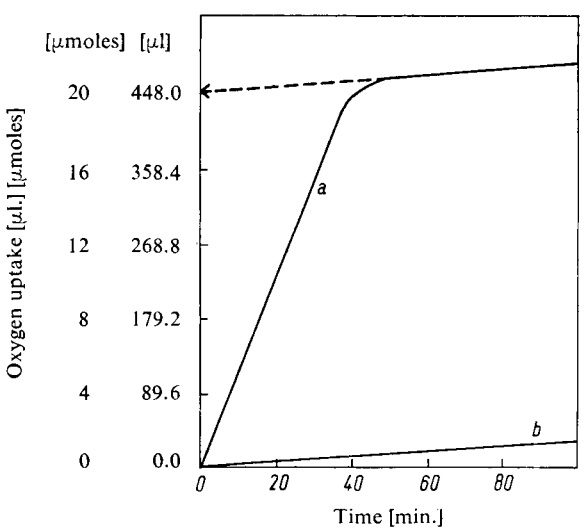

Fig. 1. Time course of the manometric determination of 40 µmoles D-lactate in the presence of 40 µmoles L-lactate (Curve a). Curve b: Oxygen uptake with 40 µmoles L-lactate.

[4] *W. W. Umbreit, R. H. Burris* and *J. F. Stauffer:* Manometric Techniques. 3rd ed., Burgess Publishing Co., Minneapolis 1957; *M. Dixon:* Manometric Methods. 3rd ed., University Press, Cambridge 1951.

The "flask constant" is the factor with which the pressure change (in mm. manometer fluid) must be multiplied to obtain the oxygen uptake in μl. The temperature at which the measurement is carried out is included in the calculation of the flask constant (refer to p. 40).
To obtain the D-lactate content of the sample, the values from column (k) are plotted against time (Fig. 1).

Figure 1 shows that the initial rapid oxygen uptake is mainly due to the oxidation of D-lactate but there is also a slower oxidation of L-lactate. By extrapolation of the slow terminal rate of L-lactate oxidation to the ordinate axis the oxygen uptake due to oxidation of D-lactate may be measured (dotted line in Fig. 1).

The D-lactate content of the sample is calculated as follows:

$$\frac{X}{11.2} \times \frac{1}{vol} = \mu\text{moles D-lactate/ml. sample} \quad (4)$$

X = μl. O_2 found by extrapolation
vol = ml. sample in manometer flask

If the factor "flask constant : 22.4" is used to convert the manometer readings into amounts of oxygen, the O_2 uptake is obtained in μmoles instead of μl. (see Fig. 1.). The concentration of D-lactate is given by the equation

$$Y \times 2 \times \frac{1}{vol} = \mu\text{moles D-lactate/ml. sample} \quad (5)$$

Y = μmoles O_2 found by extrapolation.
It is preferable to divide the flask constant by 11.2 instead of 22.4 as it is not then necessary to multiply by 2 in equation (5). To convert μmoles to mg. D-lactate multiply by 0.09 (1 μmole lactic acid = 90 μg. = 0.09 mg.).

Interfering Substances

The acetone powder is suspended in a buffer of high molarity, so that small amounts of acid or alkali in the sample do not interfere with the determination. The effect of several well-known enzyme inhibitors has been examined (Table 2).

Table 2. Effect of L-lactate and inhibitors on the determination of D-lactate

Sample	μmoles D-lactate found
10 μmoles D-lactate (potassium salt)	9.8
+ 100 μmoles NaF	9.9
+ 20 μmoles Na iodoacetate	9.7
+ 35 μmoles Na arsenate	10.3
+ 100 μmoles Na arsenite	10.0
+ 25 μmoles K-L-lactate	10.1

Specificity

The following compounds (10 μmoles or more) cause no oxygen uptake under the standard conditions: D-arabinose, D-glucose, D-ribose, D-xylose, D-lyxose, L-arabinose, ethanol, 3-keto-butan-2-ol, glycerol, glycylglycine, glycine, methylglyoxal, Na-acetate, K-citrate, K-malate (DL), K-oxaloacetate, K-propionate, Na-pyruvate.

Apart from D-lactate, L-lactate and D-glucono-δ-lactone are also oxidized (Table 3).

Table 3. Relative rates of oxygen uptake with L-lactate and D-glucono-δ-lactone in the D-lactate assay

Sample	Oxygen uptake (D-lactate = 100)
20 μmoles potassium-D-lactate	100
10 μmoles potassium-L-lactate	3
25 μmoles potassium-L-lactate	3
20 μmoles D-glucono-δ-lactone	1

Other Methods for the Determination of D-Lactate

D-Lactate can also be estimated with a purified enzyme from yeast according to *Labeyrie* et al.[1]. The determination of the pyruvate formed from D-lactate according to equation (1), with the DPN-dependent lactic dehydrogenase (reaction product: L-lactate) has not yet been examined.