58. CRYSTALLINE LACTIC DEHYDROGENASE FROM HEART MUSCLE

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(*Received 10 January 1940*)

THE reduction of methylene blue by lactic acid, or in other words, the dehydrogenation of lactic acid in the presence of extracts from animal tissues, is now known to be catalysed by a system composed of two enzymes (lactic dehydrogenase and diaphorase flavoprotein) and cozymase [Dewan & Green, 1938; Euler & Hellström, 1938]. The chemical reactions involved in this process may be written [Corran *et al.* 1939]:

(1) Lactic acid + cozymase $\rightleftharpoons$ pyruvic acid + reduced cozymase.
(2) Reduced cozymase + flavoprotein $\rightarrow$ cozymase + reduced flavoprotein.
(3) Reduced flavoprotein + methylene blue $\rightarrow$ flavoprotein + leucomethylene blue.

Reactions (2) and (3) have been described in an earlier paper [Corran *et al.* 1939] and it was shown that the flavoprotein which catalyses these reactions can be isolated from animal tissues [Straub, 1939].

Reaction (1) is catalysed by an enzyme, which can be extracted with distilled water from the tissues. The equilibrium of the reversible reaction (1) is very much in favour of lactic acid formation [Euler *et al.* 1937]. Its action is similar to that of alcohol dehydrogenase (acetaldehyde reductase of Negelein & Wulff [1937]), an enzyme, isolated from yeast, which catalyses the reduction of cozymase by ethyl alcohol and the oxidation of reduced cozymase by acetaldehyde. Our enzyme can therefore be considered either as lactic dehydrogenase or as pyruvic reductase.

But its most important function is the part it plays in the lactic fermentation, being the enzyme which catalyses the last step of lactic fermentation, i.e. the reduction of pyruvic acid to lactic acid.

In order to study the kinetics of these enzymic reactions it seemed desirable to isolate the components. In the present paper the isolation of lactic dehydrogenase in crystalline form is described.

Catalytic test

The purity of the enzyme preparations was determined throughout this work by the "lactic test" as devised by Green & Brosteaux [1936]. This test consists in the measurement of the O_2 uptake which occurs when lactic dehydrogenase is added to a mixture of lactic acid, cozymase, methylene blue, strong cyanide and flavoprotein. The purified flavoprotein was prepared from bullocks' hearts according to the method described in an earlier paper [Straub, 1939]. If any one of these ingredients was omitted no O_2 uptake could be detected. The test can be used as a measure of the quantity of lactic dehydrogenase only if care is taken to make the latter the limiting factor. All the other reactants are therefore taken in large excess and only small O_2 uptakes are measured, e.g. when using

the amounts of ingredients given in Table 1, the O_2 uptake should not exceed 40 μl. per 10 min. Below this value, the rate of O_2 uptake is proportional to the amount of lactic dehydrogenase added.

The Q_{O_2} which can be calculated from the data given in Table 1 cannot be regarded as an absolute measure of the enzyme activity under normal conditions for various reasons, amongst which the most important is the presence of strong cyanide. A detailed study of the activity of the enzyme under more physiological conditions will follow in another paper.

Preparation of the enzyme

(1) *Extraction and adsorption on Ca-phosphate.* Four bullocks' hearts are freed from fat and minced through an ordinary mincer. 5·5 kg. of the mince are mixed with 16·5 l. of ice-cold distilled water and gently stirred for 15–20 min. The fluid is separated from the muscle particles by squeezing through a thin cloth. The water extract, which contains the enzyme, is now mixed with 3 l. of a $Ca_3(PO_4)_2$ gel (freshly prepared, neutralized and washed with tap and distilled water 5–6 times). The amount of Ca-phosphate added is 5 g. for each l. of extract, i.e. 82 g. in the present case. After mixing thoroughly, the Ca-phosphate, which contains the enzyme, is centrifuged off in a Sharples centrifuge. From this gel the enzyme is eluted by mixing it with 3·5 l. of $M/5$ phosphate buffer pH 7·2. The Ca-phosphate is centrifuged off and discarded.

(2) *First fractionation with* $(NH_4)_2SO_4$. To the eluate (3·2 l.), after it has cooled down to 10°, 1250 g. of $(NH_4)_2SO_4$ are added (0·6 saturated). The precipitate is filtered off through fluted filters in the cold (4–6 hr.). The clear red filtrate is discarded and the precipitate dissolved in 400 ml. of $M/10$ pH 7·2 phosphate buffer, giving 480 ml. of a brownish solution of the enzyme.

(3) *First precipitation with acetone at 13°.* To 480 ml. of this solution, cooled to 0°, 290 ml. of very cold (−15°) acetone are added. The temperature rises to +13° and the mixture is kept at this temperature for 10 min. The precipitate is centrifuged down in cooled tubes and the fluids (two layers) are discarded.

(4) *Second precipitation with* $(NH_4)_2SO_4$. The precipitate is suspended in 200 ml. of 0·3 saturated ammonium sulphate solution, when the great bulk of denatured protein does not redissolve. The undissolved part is centrifuged off in cooled tubes at 0°. From the solution, which contains the enzyme, the acetone is removed by vacuum distillation. The sulphate content of the 190 ml. of solution is 0·32 saturated. A further 21·6 g. of ammonium sulphate are dissolved in it (0·5 saturated). The precipitate which is formed contains the enzyme and it is filtered off through a fluted filter. On dissolving the precipitate in distilled water, 55 ml. of a slightly yellow solution are obtained.

(5) *Second precipitation with acetone at 18°.* After this solution has been cooled to 0°, 33 ml. of acetone at −15° are added. After 10 min. at 18° the precipitate is centrifuged off at 0° and redissolved in 25 ml. 0·3 saturated ammonium sulphate solution. Without removing the insoluble proteins, the mixture is dialysed against 1 l. of 0·3 saturated ammonium sulphate at 0° for 12 hr. It is then centrifuged, giving 22 ml. of a clear, almost colourless solution.

(6) *Crystallization.* By the addition of 1·32 g. $(NH_4)_2SO_4$ to the 22 ml. of solution obtained in the previous step, the salt concentration is brought to 0·4 saturation and the precipitate is rapidly centrifuged off. To the clear supernatant fluid a saturated solution of ammonium sulphate is added drop by drop. When the concentration of ammonium sulphate reaches 0·5 saturation, the crystallization is complete. The precipitate is centrifuged off at 10,000 r.p.m.

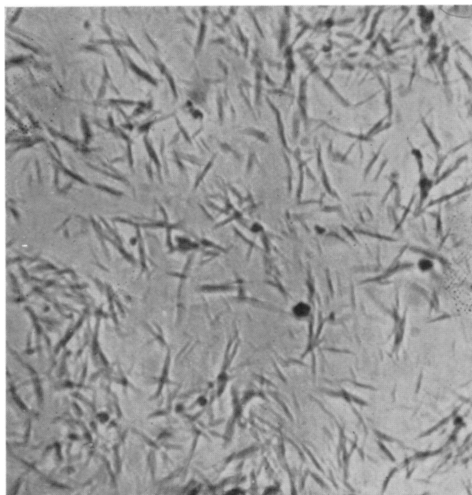

Fig. 1.

Fig. 2.

Crystalline lactic dehydrogenase. ×540.

Table 1. *Dehydrogenation of lactic acid, catalysed by different lactic dehydrogenase preparations*

In each manometer vessel are present: 0·9 ml. of a 0·4% methylene blue solution, 0·3 ml. of 10% Na lactate, 0·2 mg. muscle flavoprotein, 0·2 ml. of a freshly prepared, neutralized $M/2$ KCN solution, 2·5 mg. of cozymase preparation (about 20% pure) and phosphate buffer pH 7·2 giving a final concentration of $M/20$. Total volume 4 ml.

Temperature: 38°. Gas space: air. Warburg manometers

Lactic dehydrogenase added to the above mixture	O_2 uptake (μl.)	
	In 10 min.	In 20 min.
None	0	0
Water extract of heart muscle mg. protein		
0·148	25·4	52·5
0·222	38·3	69·0
Crystalline enzyme mg. protein		
0·0024	24·0	47·4
0·0036	35·5	66·8

The crystals are very fine needles, which tend to form clusters. Their refractive index differs so slightly from that of the ammonium sulphate solution that it is extremely difficult to get a good photograph of them. If a drop of methylene blue solution is added, the crystals take up the dye very strongly, remaining unchanged in form. Fig. 1 shows the crystals themselves and Fig. 2 shows them when stained with methylene blue.

The crystals dissolve readily in water, giving a perfectly clear and colourless solution. Recrystallization does not change their activity and the form and size of the crystals remain the same after three recrystallizations.

If a solution of the crystalline enzyme is dialysed until free from salts, the enzyme is inactivated and it is precipitated as a denatured protein, which will not dissolve again even in the presence of salts. However, if kept in 0·1 saturated ammonium sulphate solution in the cold, the activity of the pure enzyme does not change in 6 weeks.

Table 2. *Yield of enzyme at various stages of purification*

	Total protein g.	Lactic dehydrogenase g.
Water extract of 5·5 kg. muscle	177	3·5
Ca-phosphate elution	26	2·1
First $(NH_4)_2SO_4$ precipitate	5·2	1·3
First acetone precipitation	2·5	0·9
Second $(NH_4)_2SO_4$ precipitate	1·1	0·65
Second acetone precipitation	—	0·46
Crystals	0·36	0·36

Non-identity of lactic and malic dehydrogenases

The lactic and malic dehydrogenases of animal tissues have been generally regarded as different enzymes [Green & Brosteaux, 1936; Green, 1936]. Das [1937] has brought forward some experimental data suggesting a possible identity of lactic and malic dehydrogenases. It is clear from the complexity of the dehydrogenating mechanism that no conclusive evidence could be expected from the study of the impure preparations.

If crystalline lactic dehydrogenase is used in the test which has been described above (for details see Table 1), even 240 μg. enzyme give no oxidation at all if

lactic acid is replaced by *l*-malic acid, whereas 2·4 μg. enzyme give a good O_2 uptake with lactic acid. If an aqueous extract of heart muscle is added to the test in place of the pure lactic dehydrogenase, then both acids are oxidized, although lactic acid is oxidized much better than *l*-malic acid.

SUMMARY

The enzyme which catalyses the reduction of cozymase by lactic acid and the oxidation of reduced cozymase by pyruvic acid has been prepared in the crystalline form. This enzyme, together with the heart flavoprotein and cozymase, forms a complete system oxidizing lactic acid. Lactic and malic dehydrogenases are not identical.

I am grateful to Prof. D. Keilin and Prof. A. Szent-Györgyi for their advice and interest. The work was done partly (in Szeged) with the help of the Josiah Macy Jr. Foundation, New York.

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