CXLVIII. IDENTITY OF LACTIC AND MALIC DEHYDROGENASES¹

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ACCORDING to Szent-Györgyi and his collaborators the C₄-dicarboxylic acids play an important catalytic role in respiration, by virtue of their alternate oxidation and reduction. An important function is attributed therein to the enzyme catalysing the dehydrogenation of malic acid into oxaloacetic acid. With regard to the possible implications it seemed to be important to decide whether this enzyme is identical with the lactic acid dehydrogenase.

The question of the identity of lactic and malic dehydrogenases has been investigated by Green & Brosteaux [1936] and Green [1936] who found that the two dehydrogenases are different. Their conclusions are chiefly based on the observation that in different enzyme preparations the two processes, the dehydrogenations of lactic and malic acids, proceed with different relative velocities.

I am able to corroborate these observations. I am, however, of the opinion that these different velocities are not caused by the difference of the two dehydrogenases but by circumstances not appreciated by Green & Brosteaux. I am led to the conclusion that both enzymes are identical. Green's results can be explained by the fact that the affinity of the enzyme for malic acid is much higher than for lactic acid, and thus at certain smaller concentrations the enzyme will be saturated in the case of the C₄-dicarboxylic acid but remains unsaturated in the case of the lactic acid. If, however, one works in each case with the maximum concentration of substrate, i.e. that required to saturate the surface of the enzyme, times of reduction become less different, as found by Green & Brosteaux [1936] and Green [1936]. The other circumstance which influences the experiment is that oxaloacetic acid, the oxidation product of malic acid, very strongly inhibits the dehydrogenation of the substrates, as has already been emphasized by Banga & Szent-Györgyi [1936]. Owing to this oxaloacetic acid inhibition, the decoloration time thus gives different indications of the intensity of hydrogen activation. This is not the case with lactic acid, the dehydrogenation of which is much less inhibited by pyruvic acid. The inhibiting action of oxaloacetic acid can be removed by coupling this substance with glutamic acid, Banga & Szent-Györgyi [1936] having found that in presence of tissue oxaloacetic acid combines at a very high rate with glutamic acid. If the experiments on the rate of dehydrogenation of malic acid are carried out in presence of glutamic acid and compared with the rate of dehydrogenation of lactic acid, maximum concentrations of substrate being used in each case, the reduction times with different preparations of enzyme are found to be equal within the limit of experimental error.

The identity of the two enzymes is also supported by the fact that no additive effect could be obtained with the two substrates.

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The fact that oxaloacetic acid, the oxidation product of malic acid, inhibits the dehydrogenations of both lactic and malic acids to approximately the same extent also strongly supports the identity of the two dehydrogenases. On the other hand, pyruvic acid, the oxidation product of lactic acid, does not inhibit the dehydrogenation of malic acid. This can be explained by the fact that the affinity of the enzyme for malic acid is much higher than for lactic acid.

The inhibitions by different substances have been tested. It has been found that both enzymes are inhibited by arsenious and iodoacetic acids. Fluoride inhibits only lactic acid dehydrogenase and not malic acid dehydrogenase under the experimental conditions.

That the two dehydrogenases should be identical is not very striking, since malic acid and oxaloacetic acid are simply carboxy-lactic and carboxy-pyruvic acid respectively.

All our enzyme preparations contained a very active fumarase which would convert fumaric and malic acid into an equilibrium mixture at a very high rate. It is thus immaterial which of these substrates is added to the enzyme preparation.

EXPERIMENTAL

The enzyme was prepared according to Banga [1935] by washing three times with equal volume of 0.9% NaCl and was finally suspended in phosphate buffer at pH 7.0.

The experiments were carried out at 37° in Thunberg tubes, the hollow stoppers being used for the dye. Each tube contained 1 ml. buffered enzyme; 0.5 ml. phosphate buffer ($M/15$; pH 7.0); 0.5 ml. coenzyme preparation; 1 ml. sodium indo-2:6-dichlorophenol-1-naphthol-2-sulphonate; x ml. substrate and $1-x$ ml. water; the total volume being 4 ml. Sets of six tubes were evacuated simultaneously with an oil pump [Szent-Györgyi, 1935]. *dl*-Lactic acid and *l*-malic acid have been used as substrates throughout this investigation.

The concentrations of the solutions generally used were as follows: sodium indo-2:6-dichlorophenol-1-naphthol-2-sulphonate (1 in 5000); arsenious acid 0.125 M ; iodoacetic acid 1.0 M ; coenzyme 1 mg. per ml.

For the sake of brevity only typical examples of the large number of experiments are given. All experiments given here were done with enzyme preparation from pigeon breast muscle unless otherwise stated.

Affinity of the enzyme

In order to examine the evidences for and against the identity of lactic and malic dehydrogenases some aspects of their kinetics have been considered. It has been found that the velocity of dehydrogenation is much slower with malic acid than with lactic acid. This is due to the fact that the inhibition of the dehydrogenation of malic acid by oxaloacetic acid (the oxidation product of malic acid) is much more marked than is the inhibition of lactic acid by pyruvic acid (the oxidation product of lactic acid). The effective concentration of oxaloacetic acid which inhibits the dehydrogenation of malic acid is about ten times less than the effective concentration of pyruvic acid which inhibits the dehydrogenation of lactic acid (Table I). Moreover with the same concentrations of oxaloacetic acid and pyruvic acid the inhibition of the malic dehydrogenase by the former was found to be much more than the inhibition of lactic dehydrogenase by the latter (Table II).

Banga & Szent-Györgyi [1936] have found that in the presence of tissue oxaloacetic acid combines very rapidly with glutamic acid. It has been found

Table I

No.	Enzyme sol. ml.	Phos-phate buffer ml.	<i>M</i> malate ml.	<i>M</i> lactate ml.	Co-enzyme ml.	Oxalo-acetic acid (0.2 mg./ml.) ml.	Pyruvic acid (1 mg./ml.) ml.	Time of de-coloration in min.	Per-centage in-hibition
1	1.0	0.5	—	—	0.5	—	—	>30.0	—
2	1.0	0.5	0.5	—	0.5	—	—	3.0	—
3	1.0	0.5	0.5	—	0.5	0.1	—	7.0	57
4	1.0	0.5	—	0.5	0.5	—	—	1.5	—
5	1.0	0.5	—	0.5	0.5	—	0.1	2.16	30

Table II

No.	Enzyme sol. ml.	Phos-phate buffer ml.	<i>M</i> malate ml.	<i>M</i> lactate ml.	Co-enzyme ml.	Oxalo-acetic acid (1 mg./ml.) ml.	Pyruvic acid (1 mg./ml.) ml.	Time of de-coloration in min.	Per-centage in-hibition
1	1.0	0.5	—	—	0.5	—	—	>30.0	—
2	1.0	0.5	0.5	—	0.5	—	—	7.0	—
3	1.0	0.5	0.5	—	0.5	0.1	—	10.5	33
4	1.0	0.5	—	0.5	0.5	—	—	4.5	—
5	1.0	0.5	—	0.5	0.5	—	0.1	5.25	14

that, if the dehydrogenation experiments are carried out in presence of glutamic acid, the times of dehydrogenation are the same within experimental error, maximum concentration of the substrates being used (Fig. 1). This is not due to substrate action of glutamic acid, for the reduction of the dye with glutamic acid alone is very slow, and to minimize this error equal amounts of glutamic acid have been added with all the concentrations of lactic acid.

It has also been observed (Fig. 1) that the "maximum" concentration of lactic acid is about five to eight times greater than the maximum concentration of malic acid. These maximum concentrations of the substrates have been found to be different in different enzyme preparations. But in all the cases the maximum concentration of lactic acid was much greater than the maximum concentration of malic acid. In some enzyme preparations slight inhibition was observed with concentration of substrates higher than the maximum, but this was not observed regularly. From these facts it appears that the affinity of the enzyme is much higher towards malic acid than towards lactic acid.

The velocity of dehydrogenation has been calculated by multiplying the inverse of the dehydrogenation time by 3000.

Fig. 1. Curve (A). Lactic acid + glutamic acid 1 mg. Curve (B). Malic acid + glutamic acid 1 mg.

Inhibition by oxaloacetic acid and pyruvic acid

The question of whether the oxidation product of one substrate inhibits the oxidation of the other substrate has also been studied; i.e. does oxaloacetic acid, the oxidation product of malic acid, inhibit the dehydrogenation of lactic acid, and conversely does pyruvic acid, the oxidation product of lactic acid, inhibit the dehydrogenation of malic acid? In the former case the lactic acid dehydrogenation was inhibited to an extent of 50–60% using the maximum concentration of lactic acid while with the same concentration of oxaloacetic acid the dehydrogenation of malic acid was also inhibited to the same extent (Table III). But pyruvic acid inhibited only the dehydrogenation of lactic acid and not the dehydrogenation of malic acid (Table IV). This inability of pyruvic acid to inhibit the dehydrogenation of malic acid can be explained by the fact, that the affinity of the enzyme is much higher for malic acid than for lactic acid.

Table III

	Percentage inhibition (mg. oxaloacetic acid)			Source of enzyme
	0.4	1.0	2.0	
Sodium lactate 0.125 <i>M</i>	55	55	55	Pigeon breast muscle
" " "	63	63	63	Pig liver
" " "	—	—	50	Pig kidney
Sodium malate 0.025 <i>M</i>	50	—	—	Pigeon breast muscle

Table IV

	Percentage inhibition Sodium pyruvate			Source of enzyme
	0.00025 <i>M</i>	0.0005 <i>M</i>	0.00075 <i>M</i>	
Sodium fumarate 0.00125 <i>M</i>	0	0	0	Pig liver
Sodium malate 0.00125 <i>M</i>	0	0	0	Pig kidney
" " "	Acceleration in several cases			Pigeon breast muscle

Enzymes from different sources

The relative rates of dehydrogenation of lactic and malic acids have also been investigated in different enzyme preparations from various sources such as pigeon breast muscle, pig kidney, pig liver and pig heart. In all these enzyme preparations the ratio of $\frac{\text{Reduction time with lactic acid}}{\text{Reduction time with malic acid}}$ has been found to lie within the range of 1.5 and 0.8, using always the maximum concentrations of the substrates (Table V).

Table V

No.	Source of enzyme	Reduction times without glutamic acid (in min.)		Reduction times with glutamic acid (in min.)		Reduction time with lactate Reduction time with malate	
		Lactate	Malate	Lactate	Malate	Without glutamic acid	With glutamic acid
1	Pigeon muscle	6.0	4.0	—	—	1.5	—
2	"	12.5	10.0	—	—	1.25	—
3	"	6.5	5.5	—	—	1.2	—
4	"	2.5	6.5	2.33	2.5	0.4	0.9
5	"	2.08	2.25	1.0	1.16	0.5	0.9
6	"	1.83	4.0	1.75	2.16	0.3	0.8
7	Pig kidney	4.5	3.0	—	—	1.5	—
8	Pig liver	6.0	4.0	—	—	1.5	—
9	Pig heart	14.0	28.0	11.5	13.5	0.5	0.9

In Exps. 4, 5 and 6 in Table V the dehydrogenation of malic acid took much longer than the dehydrogenation of lactic acid. But by adding glutamic acid the times of dehydrogenation with lactic and malic acid became equal within the limits of the accuracy of the experiments. In other experiments the enzymes were not thoroughly washed, and as a result some donator already present in the enzyme probably combined with the oxaloacetic acid formed during the dehydrogenation of malic acid. Uniform results giving longer dehydrogenation time with malic acid have been found in many enzyme preparations, which had been thoroughly washed, and in all these cases the dehydrogenation times with malic and lactic acids became equal when glutamic acid was added. From these results it appears that the two enzymes are identical.

Dual destruction

During this investigation whenever an enzyme preparation was for any reason inactive towards malic acid its activity towards lactic acid was also destroyed. In none of the cases was there found a case either of separation or of individual destruction of the enzyme.

Inhibitors

It seemed to be of interest also to study the effect of some inhibitors on the enzymes. All these inhibition experiments have been carried out with enzyme prepared from pigeon breast muscle, pig kidney and pig liver. Only those with pigeon muscle enzyme are given here.

Arsenious acid. Sodium arsenite inhibits both lactic and malic acid dehydrogenases to the same extent (Table VI).

Table VI

No.	Enzyme sol. ml.	Phosphate buffer ml.	Coenzyme ml.	M/5 malate ml.	M lactate ml.	M/8 sodium arsenite ml.	Time of de-coloration in min.	Percentage inhibition
1	1.0	0.5	0.5	—	—	—	>30.0	—
2	1.0	0.5	0.5	0.5	—	—	1.5	—
3	1.0	0.5	0.5	0.5	—	0.3	6.5	77
4	1.0	0.5	0.5	—	0.5	—	2.25	—
5	1.0	0.5	0.5	—	0.5	0.3	8.0	72
6	1.0	0.5	0.5	—	—	—	>30.0	—

Iodoacetic acid. Sodium iodoacetate also inhibits both the dehydrogenases (Table VII).

Table VII

No.	Enzyme sol. ml.	Phosphate buffer ml.	Coenzyme ml.	M/5 malate ml.	M lactate ml.	M iodo-acetic acid ml.	Time of de-coloration in min.	Percentage inhibition
1	1.0	0.5	0.5	—	—	—	>30.0	—
2	1.0	0.5	0.5	0.5	—	—	7.0	—
3	1.0	0.5	0.5	0.5	—	0.5	14.5	51
4	1.0	0.5	0.5	—	0.5	—	15.0	—
5	1.0	0.5	0.5	—	0.5	0.5	>30.0	c. 100
6	1.0	0.5	0.5	—	—	—	>30.0	—

Sodium fluoride only inhibits the lactic dehydrogenation and not the malic dehydrogenation under the experimental conditions (Table VIII).

Table VIII

No.	Enzyme sol. ml.	Phosphate buffer ml.	Co-enzyme ml.	<i>M</i> /5 fumarate ml.	<i>M</i> lactate ml.	Sodium fluoride (2 mg./ml.) ml.	Time of decoloration in min.	Percentage inhibition
1	1.0	0.5	0.5	—	—	—	>30.0	—
2	1.0	0.5	0.5	0.5	—	—	1.5	—
3	1.0	0.5	0.5	0.5	—	0.5	1.5	0
4	1.0	0.5	0.5	—	0.5	—	2.0	—
5	1.0	0.5	0.5	—	0.5	0.5	4.08	49
6	1.0	0.5	0.5	—	—	0.5	>30.0	—

Method of competition

When the enzyme is treated simultaneously with maximum concentrations both of lactic and malic acids, the dehydrogenation time (with both the substrates added together) lies between the respective dehydrogenation times with each substrate added separately. Several experiments were done with different enzyme preparations from pigeon breast muscle, pig kidney and pig liver. In none of the cases was any summation or additive effect observed. The two substrates thus appear to be competing for the same enzyme (Tables IX and X).

Table IX

No.	Enzyme sol. ml.	Phosphate buffer ml.	Coenzyme ml.	<i>M</i> fumarate ml.	<i>M</i> lactate ml.	Time of decoloration in min.
1	1.0	0.5	0.5	—	—	>30.0
2	1.0	0.5	0.5	0.2	—	10.0
3	1.0	0.5	0.5	0.3	—	10.0
4	1.0	0.5	0.5	—	0.4	12.5
5	1.0	0.5	0.5	—	0.5	12.5
6	1.0	0.5	0.5	0.2	0.4	10.5

Table X

No.	Enzyme sol. ml.	Phosphate buffer ml.	Coenzyme (1 mg./ml.) ml.	<i>M</i> /5 malate ml.	<i>M</i> lactate ml.	Time of decoloration in min.
1	1.0	0.5	0.5	—	—	>30.0
2	1.0	0.5	0.5	0.4	—	2.0
3	1.0	0.5	0.5	0.5	—	2.0
4	1.0	0.5	0.5	—	0.3	1.16
5	1.0	0.5	0.5	—	0.4	1.16
6	1.0	0.5	0.5	0.4	0.3	1.66

Adsorption with kaolin

Green & Brosteaux [1936] found that after adsorption of the enzyme with kaolin the dehydrogenations of lactic and malic acids proceeded with different velocities. They put forward this as evidence against the identity of the enzymes.

Table XI

Coenzyme was added in all these experiments.

No.	Enzyme sol. before adsorption ml.	Phosphate buffer ml.	Enzyme sol. after adsorption ml.	Glutamic acid sol. (10 mg./ml.) ml.	<i>M</i> /5 malate ml.	<i>M</i> lactate ml.	Time of decoloration in min.
1	1.0	0.5	—	0.1	—	—	>60.0
2	1.0	0.5	—	0.1	0.5	—	11.5
3	1.0	0.5	—	0.1	—	0.5	13.5
4	—	0.5	1.0	0.1	0.5	—	32.0
5	—	0.5	1.0	0.1	—	0.5	32.0

Table XII

No.	Enzyme sol. before adsorption ml.	Phosphate buffer ml.	Enzyme sol. after adsorption ml.	Coenzyme sol. ml.	M/5 malate ml.	M lactate ml.	Time of decoloration in min.
1	1.0	0.5	—	0.5	0.5	—	28
2	1.0	0.5	—	0.5	—	0.5	14
3	—	0.5	1.0	0.5	0.5	—	95
4	—	0.5	1.0	0.5	—	0.5	36

Their experiments were repeated. By adding glutamic acid, the reduction rates with both lactic and malic acid were found to be equal both before and after adsorption (Tables XI and XII).

The enzyme for this purpose was prepared according to Green & Brosteaux [1936] from pig heart and a portion was adsorbed three times with kaolin.

Coenzyme

It has been found that cozymase free from adenylic acid ($ACo=600,000$) which was kindly supplied by Prof. Hans von Euler activates the dehydrogenation of malic acid (Fig. 2).

Fig. 2.

SUMMARY

1. Lactic and malic dehydrogenases have been found in pigeon breast muscle, pig kidney, pig liver and pig heart.
2. The effective concentration of oxaloacetic acid which inhibits malic dehydrogenase is much less than the effective concentration of pyruvic acid which inhibits lactic dehydrogenase.
3. With the same concentration of both oxaloacetic acid and pyruvic acid the inhibition of malic dehydrogenase is much greater by the former than the inhibition of lactic dehydrogenase by the latter.

4. The relative velocities of dehydrogenation of lactic and malic acids are almost equal in all enzyme preparations when glutamic acid is added with malic acid to combine with the oxaloacetic acid which is formed during the dehydrogenation of malic acid.

5. The maximum concentration of malic acid is much less than the maximum concentration of lactic acid.

6. Oxaloacetic acid inhibits the dehydrogenations of both malic and lactic acids while pyruvic acid inhibits only the dehydrogenation of lactic acid and not that of malic acid.

7. Arsenious acid and iodoacetic acid inhibit both dehydrogenations, but fluoride inhibits only lactic dehydrogenase and not malic dehydrogenase.

8. No summation or addition has been found with lactic and malic acid added together.

9. The enzymes were not separated by adsorption with kaolin.

10. Cozymase free from adenylic acid activates malic dehydrogenase.

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