CXLIX. STUDIES ON THE INHIBITION OF THE SUCCINIC AND LACTIC- MALIC DEHYDROGENASES¹

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THE theory of Szent-Györgyi and his collaborators that succinic and malic acids play important catalytic roles in the normal respiration of animal tissues by virtue of their alternate oxidation and reduction led to the present investigation, which deals with the mutual inhibition of these oxidation-reduction processes by the products of their oxidation and reduction. Evidence has been given in the preceding paper for the identity of the malic and lactic acid dehydrogenating enzymes and this enzyme was therefore also studied along these lines. Since, furthermore, inhibiting effect of malonic acid on respiration is an important feature of Szent-Györgyi's theory a study of this substance was also undertaken.

EXPERIMENTAL

The dehydrogenation experiments were carried out by the method described in the previous paper. Sodium indo-2:6-dichlorophenol-1-naphthol-2-sulphonate has been used in all the experiments excepting in the case of succinic acid where 2:6-dibromophenolindophenol has been used as the dye.

The hydrogenation experiments were carried out in an apparatus especially designed for the purpose using neutral red (1 in 5000) as the dye. This was reduced *in vacuo* by neutralized sodium hydrosulphite solution in slight excess and added to the enzyme-substrate mixture. The first appearance of the red colour was taken as the hydrogenation time in all the experiments.

The details of the apparatus are given in Fig. 1. It consists of 6 tubes (*L*) which can be attached to the main apparatus by ground joints. These tubes are meant for the enzyme-substrate mixture. The dye is to be stored in *B*. *D* is a calibrated container for the reduced dye and *H* is a capillary joined to *D* by a stopcock *E* and passing through the stopper *F*. The capillary *H* can be rotated so that the measured amount of reduced dye from *D*

Fig. 1.

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can be dropped into each of the tubes *L*. *M* is meant for neutralized hydrosulphite which can be dropped into the dye stored in the vessel *B* by turning the stopper *A* very slowly. *G* is the stopper for evacuating the whole apparatus.

The tubes containing the enzyme-substrate mixture are joined to the main apparatus and the stopcock *A* is closed. The whole apparatus can then be evacuated through the stopcock *G* keeping the stopcock *E* open to both *D* and *H*. The apparatus is then evacuated for about 4 min. by an oil pump and the neutralized solution of sodium hydrosulphite is dropped from *M* into the dye stored in *B* by turning the stopper *A* very slowly, taking care that no air bubble enters *B* and that the dye is a little over-reduced. The stop-cocks *A* and *E* are then closed and the reduced dye from *B* is dropped into the reservoir *D* by rotating the vessel *B* about the ground joint *C*. One of the tubes *L* should be without enzyme-substrate mixture and be used for labelling the dye against the calibrations in the reservoir *D*. Then 1 ml. of the reduced dye is dropped into the respective tubes *L* and the apparatus is immersed in a water-bath at 37°.

Accuracy of the apparatus

This apparatus has been found to give fairly accurate results, the error never being more than 20%. A typical series of experiments is given in Table I.

Table I

No.	Enzyme sol. ml.	Phosphate buffer ml.	<i>M</i> /10 pyruvate ml.	Time of decoloration in min.
1	1.0	1.0	0.5	7.0
2	1.0	1.0	0.5	7.0
3	1.0	1.0	0.5	8.0
4	1.0	1.0	0.5	7.5
5	1.0	1.0	0.5	7.5

Inhibition experiments

Inhibitions of both dehydrogenation and hydrogenation by their respective reaction products have been studied. In all these experiments the object has been to determine the concentration of substance which gives 50% inhibition. The results are the averages of at least 3 experiments.

Succinic dehydrogenase (fumarase-free) was prepared by Lehman's [1929] method. 100 g. of fat-free horse muscle were minced in a Latapie mincer and washed with water till free from haemoglobin. The washed muscle was then stirred with warm water (50°) for about 5 min. taking care to keep the temperature constant and to squeeze immediately after through muslin in order to make it relatively free from fumarase. The pressed tissue was again washed with cold water, ground with sand and 100 ml. phosphate buffer (*pH* 7.0) and ultimately pressed through muslin. The phosphate extract was used as the enzyme preparation in all the experiments described below.

Inhibition of dehydrogenation of succinic acid

Succinic dehydrogenase is very strongly inhibited by oxaloacetic acid and malonic acid whilst the inhibition by fumaric acid is very weak (Table II). It is not inhibited by malic or lactic acid, even when added in quantity double that of the succinic acid.

Table II

No.	Buffered enzyme ml.	M/5 succinate ml.	Amount of substance for 50 % inhibition
1	1.0	0.5	0.01 mg. oxaloacetic acid
2	1.0	0.5	0.8 mg. malonic acid
3	1.0	0.5	10.0 mg. fumaric acid

Inhibition of the hydrogenation of fumaric acid

The hydrogenation of fumaric acid is less inhibited by oxaloacetic acid and malonic acid than is the dehydrogenation of succinic acid, but the inhibition by succinic acid is greater than the inhibition of dehydrogenation by fumaric acid (Table III). It is, however, not inhibited by either malic or lactic acid when added in quantity equal to that of the fumaric acid.

Table III

No.	Buffered enzyme ml.	M/5 fumarate ml.	Amount of substance for 50 % inhibition
1	1.0	0.5	0.3 mg. oxaloacetic acid
2	1.0	0.5	4.0 mg. malonic acid
3	1.0	0.5	2.4 mg. succinic acid

Lactic-malic dehydrogenase. The enzyme has been prepared from pigeon breast muscle as described in the previous paper.

Inhibition of dehydrogenation of malic acid

Malic acid dehydrogenase is very strongly inhibited by oxaloacetic acid, whereas the inhibition by malonic acid is very slight (Table IV). The inhibition by pyruvic acid has been discussed in the previous paper. These experiments with malic acid have been done with "maximum" concentration of the substrate, i.e. that required to saturate the surface of the enzyme.

Table IV

No.	Buffered enzyme ml.	M/5 malate ml.	Amount of substance for 50 % inhibition
1	1.0	0.5	0.02 mg. oxaloacetic acid
2	1.0	0.5	24.0 mg. malonic acid

Inhibition of hydrogenation of oxaloacetic acid

The inhibition of hydrogenation of oxaloacetic acid by malic acid is much less than the inhibition of dehydrogenation of malic acid by oxaloacetic, but the hydrogenation is more markedly inhibited by malonic acid than is the dehydrogenation. Lactic acid, however, failed to inhibit the reaction, which can be explained by the higher affinity of the enzyme towards malic than towards lactic acid (Table V).

Table V

No.	Buffered enzyme ml.	M/5 oxaloacetate ml.	Amount of substance for 50 % inhibition
1	1.0	0.5	3.5 mg. malic acid
2	1.0	0.5	5.0 mg. malonic acid

Inhibition of dehydrogenation of lactic acid

Lactic acid dehydrogenase is strongly inhibited by pyruvic acid and malonic acid. In the previous paper it has been shown that it is also inhibited by oxaloacetic acid to an equal extent (Table VI). These experiments with lactic acid have also been done with the maximum concentration of the substrate.

Table VI

No.	Buffered enzyme ml.	<i>M</i> lactate ml.	Amount of substance for 50 % inhibition
1	1.0	0.5	0.3 mg. pyruvic acid
2	1.0	0.5	0.5 mg. malonic acid

Inhibition of the hydrogenation of pyruvic acid

The hydrogenation of pyruvic acid has been found to be inhibited almost equally by malonic and lactic acids (Table VII).

Table VII

No.	Buffered enzyme ml.	<i>M</i> /10 pyruvate ml.	Amount of substance for 50 % inhibition
1	1.0	0.5	3.0 mg. lactic acid
2	1.0	0.5	4.0 mg. malonic acid

Table VIII

	0.3 mg. pyruvic acid (50 %)	
Lactic acid	↔	pyruvic acid
	3.0 mg. lactic acid (50 %)	
	0.5 mg. malonic acid (50 %)	
Lactic acid	↔	pyruvic acid
	4.0 mg. malonic acid (50 %)	
	0.02 mg. oxaloacetic acid (57 %)	
Malic acid	↔	oxaloacetic acid
	3.5 mg. malic acid (50 %)	
	24.0 mg. malonic acid (50 %)	
Malic acid	↔	oxaloacetic acid
	5.0 mg. malonic acid (50 %)	
	10.0 mg. fumaric acid (50 %)	
Succinic acid	↔	fumaric acid
	2.4 mg. succinic acid (53 %)	
	0.8 mg. malonic acid (50 %)	
Succinic acid	↔	fumaric acid
	4.0 mg. malonic acid (50 %)	
	0.01 mg. oxaloacetic acid (50 %)	
Succinic acid	↔	fumaric acid
	0.3 mg. oxaloacetic acid (40 %)	
Succinic acid	→	fumaric acid
	(Not inhibited by malic or lactic acid)	
Succinic acid	←	fumaric acid
	(Not inhibited by malic or lactic acid)	

A summary of the results is given in Table VIII where the vertical arrows indicate the inhibition and the horizontal arrows pointing to right and left indicate dehydrogenation and hydrogenation respectively. The figures within brackets indicate percentage inhibitions.

DISCUSSION

The inhibitions described in the experimental part allow certain conclusions regarding the theory of hydrogen transference by C_4 -dicarboxylic acids. At the beginning of these studies Gözsy & Szent-Györgyi [1934] presented evidence in favour of the assumption that the C_4 -dicarboxylic acids performed their function by their lowest step of oxidation, that is the change succinate $\rightarrow$ fumarate. The main evidence was based upon experiments with malonic acid, which inhibits respiration and also inhibits the succinic dehydrogenation but not the malic dehydrogenation. The theory that hydrogen was transferred by the change succinate $\rightarrow$ fumarate, however, was given up, since it was found that the respiration inhibited by malonate could be revived by the addition of a small quantity of fumarate. After the addition of this substance the respiration proceeded undisturbed in spite of the presence of malonate. This led to the assumption that the C_4 -dicarboxylic acids exert their catalytic function by a higher step of oxidation, *viz.* the change malic $\rightarrow$ oxaloacetic acid, which was practically insensitive to malonate. This latter theory led to the discovery of the strikingly rapid reduction of oxaloacetic acid by the respiring tissue. Fumaric acid is not reduced by the tissue at a considerable velocity, which also argues against the possible catalytic function of the succinic $\rightarrow$ fumaric acid step. Neither of these theories can, however, explain all the phenomena, e.g. the function of fumarase. The succinate theory gives no explanation of the striking reduction of oxaloacetate, whilst the malic theory gives no explanation of the existence and exceptional qualities of the succinic dehydrogenase. The whole of the phenomena observed can only be explained by a combined theory in which both processes are involved.

The data presented in this paper invalidate the arguments brought forward against the succinate-fumarate theory showing that adsorption of fumarate is less inhibited by malonate than is the adsorption of succinate. To appreciate this point, we have to bear in mind that, of the four processes involved, the oxidation of succinate and the reduction of oxaloacetate are very fast compared with the reverse processes, the reduction of fumarate and oxidation of malate. So at any moment most of the catalyst will be found in the form of its malate-fumarate equilibrium mixture (equilibrium being maintained by the fumarase). The time during which a succinate or an oxaloacetate molecule can exist as such will be very brief. The form in which the catalyst is adsorbed on the succinic dehydrogenase will be fumarate. There will be thus a considerable difference in the oxidation of succinate added as such to the muscle (strongly inhibited by malonate) and that of succinate present in the tissue and playing by its oxidation and reduction the role of a catalyst. It will be adsorbed as fumarate and, if once adsorbed, will be converted only for a very short time into succinate, which at the moment of its formation is in an adsorbed condition and does not compete with malonic acid, and will be reoxidized to fumaric acid at a very high rate.

One further circumstance to be borne in mind is the very low normal concentration of the catalyst. This is approximately 10 mg. per 100 ml. in muscle. If, as happens in respiration experiments, the tissue is suspended in ten times

its volume of fluid, the concentration decreases to 1 mg. per 100 ml. So even the addition of a very small quantity of fumaric acid, say 2 mg., means a 50-fold increase of its concentration.

My experiments show that the reduction, i.e. adsorption of fumaric acid, is distinctly less inhibited by malonate than the oxidation, i.e. adsorption of succinic acid. At a very low concentration of the catalyst both adsorptions will be inhibited by a small concentration of malonate. If, however, the concentration of the catalyst is increased by addition of fumarate, it is possible that malonate will be unable to compete efficiently with the adsorption of fumarate though, as experiments show that malonate can inhibit the oxidation of succinate, there is a possibility that malonate cannot inhibit the reoxidation of succinate, adsorbed as fumarate and formed in an adsorbed condition on the enzymic surface, where (owing to its rapid oxidation) the time of its existence will be too short to give full play for the competition of malonate.¹

There is sufficient reason to suppose that in its relation to malonate there is a distinct difference between the succinate, which is, on the one hand, added to the tissue and has in its adsorption to compete with malonate, and the succinate which, on the other hand, is formed by the reduction of fumarate and is therefore at the moment of its formation in an adsorbed condition and so capable of exerting its catalytic function without competing with the malonate present.

According to the observations of this paper, malonate inhibits the reduction of fumaric acid much less markedly than it inhibits the oxidation of succinate, and I therefore think that the restoration of malonate-inhibited respiration by fumarate is no argument against the catalytic function of succinic dehydrogenase and of the step succinate-fumarate.

My experiments also show that the inhibition of reduction of fumarate to succinate by succinic acid is much more marked than the inhibition of oxidation of succinate by fumarate. This shows that the reduction of added fumarate must very soon be inhibited by accumulation of succinate. No considerable accumulation of succinate can therefore be expected and the reaction must be greatly slowed down at its early stages. So the failure of the tissue to reduce added fumarate in quantity at a considerable rate cannot be used as an argument against the possible catalytic function of the process succinate-fumarate.

Another point worth mentioning is the possible bearing of these observations on the Pasteur reaction. According to the most recent theories lactic fermentation is in its essence a transfer of the hydrogen from triose to pyruvic acid. According to Szent-Györgyi's theory oxidation of the same substance would mean the transference of its hydrogen to oxaloacetic acid. I have shown that the affinity of the enzyme, when activating both substrates, is much greater towards oxaloacetic acid than towards pyruvic acid. So if in presence of oxygen oxaloacetic acid is formed, the latter would have to become the main acceptor of the hydrogen and oxidation would proceed. If no oxygen is present, pyruvic acid would have to be the acceptor and fermentation would proceed.

One more point worth mentioning is the much greater affinity of the enzyme for the oxidized forms of its substrates (i.e. pyruvic acid and oxaloacetic acid) than for their reduced forms (i.e. lactic and malic acids). This is evident from the comparatively strong inhibition of the dehydrogenation by pyruvic and oxaloacetic acids. It seems that the conversion of the CHOH group into the CO group

¹ Naturally a small part of succinate will be replaced by malonate even under these conditions and will be inhibited in its readsorption and oxidation. Possibly this is the reason for the accumulation of succinic acid in presence of malonate described by Gözsy [1935].

greatly increases the adsorption. Since oxaloacetic acid is reduced at a very high rate it will not interfere with the further oxidation of malic acid. Pyruvic acid, however, is not removed at an equally high rate and so it may be concluded that the main function of the enzyme in regard to the C_3 acids is related to hydrogenation (reduction of pyruvic acid) rather than to dehydrogenation (oxidation of lactic acid), which is in accordance with the modern theory of fermentation.

The last point worth noting is the importance of doing experiments in absence of the reaction product when comparing the relative activities of the enzyme towards different substrates.

SUMMARY

1. The inhibition of dehydrogenation of lactic acid by pyruvic acid is stronger than the inhibition of hydrogenation of pyruvic acid by lactic acid.

2. Malonic acid inhibits dehydrogenation of lactic acid more strongly than hydrogenation of pyruvic acid.

3. The inhibition of dehydrogenation of malic acid by oxaloacetic acid is stronger than the inhibition of hydrogenation of oxaloacetic acid by malic acid.

4. Malonic acid inhibits hydrogenation of oxaloacetic acid whilst the dehydrogenation of malic acid is inhibited only by a large amount of malonic acid.

5. The inhibition of dehydrogenation of succinic acid by fumaric acid is much weaker than the inhibition of hydrogenation of fumaric acid by succinic acid.

6. Malonic and oxaloacetic acids inhibit dehydrogenation of succinic acid more strongly than hydrogenation of fumaric acid.

7. Malic or lactic acid failed to inhibit both dehydrogenation of succinic acid and hydrogenation of fumaric acid.

8. The bearing of these results on the theory of tissue respiration is discussed.

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