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Some Properties of the Malic Enzyme of Pigeon Liver

1. CONVERSION OF MALATE INTO PYRUVATE

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The malic enzyme, first described in pigeon liver by Ochoa, Mehler & Kornberg (1948), catalyses the reaction:

where TPN^+ and TPNH stand for the oxidized and reduced forms of triphosphopyridine nucleotide. The enzyme is thought to play a role in the synthesis of glycogen from lactate and pyruvate and to be one of the factors controlling the supply of oxaloacetate in the liver (Krebs, 1954; Hiatt, Goldstein, Lareau & Horecker, 1958). Although the coenzyme requirements of the enzyme are firmly established relatively little is known about the kinetics and the effects of inhibitors. Rutter & Lardy (1958) have examined the effects of pH and cations on the conversion of malate into pyruvate.

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A number of carboxylic acids, such as malonic acid, tartronic acid, mesoxalic acid, D- and meso-tartaric acid, oxalic acid, fluoromalic acid and fluorocitric acid, cause an accumulation of acetoacetate in respiring liver preparations (Edson, 1936; H. A. Krebs, unpublished work). Since ketogenesis may be related to the supply of oxaloacetate, the action of the above-mentioned acids might be due to the inhibition of either the 'oxaloacetate-synthesizing enzyme' (Utter & Kura-hashi, 1955), which catalyses the reaction

(where GDP and GTP stand for guanosine di- and tri-phosphate and IDP and ITP for inosine di- and tri-phosphate), or malic enzyme, both of which lead to the formation of oxaloacetate from the products of glycolysis. The action of the ketogenic

acids on these two enzymes was therefore investigated.

The first paper of this series is concerned with a study of the kinetics of the decarboxylation of malate to pyruvate and of the effects of the ketogenic carboxylic acids on this reaction. Their effects on the synthesis of malate by malic enzyme and on the oxaloacetate-synthesizing enzyme are reported in subsequent papers (Stickland, 1959*a, b*).

EXPERIMENTAL

Preparation of enzyme. The enzyme was prepared from pigeon liver by the method of Ochoa (1955), modified in the first stages by Dr D. D. Davies (personal communication). The modifications consisted of the omission of the acetone precipitation; instead of this, some of the inactive cell fractions were removed by centrifuging. One of the samples of purified malic enzyme used was kindly given by Dr D. D. Davies.

Pigeon livers (150 g.) were homogenized in a stainless-steel homogenizer of the Potter-Elvehjem type in 2 vol. of cold 0.9% KCl. The homogenate was centrifuged at 650 *g* for 2 min.; the residue was suspended in 0.67 vol. of cold 0.9% KCl and re-centrifuged (650 *g* for 2 min.). The combined supernatants (300 ml.) contained practically no material which could be sedimented at 2500 *g* in 60 min. and were used for the next step. Ammonium sulphate sufficient for full saturation (210 g.) was added slowly at 1° to the supernatants. The precipitate was centrifuged off, dissolved in 20 ml. of 0.02M-potassium phosphate buffer, pH 7.4, and dialysed for 24 hr. at 5° against the same buffer. The phosphate buffer was changed several times and stirred by a stream of air bubbles. A slight precipitate in the dialysed solution was centrifuged off and the supernatant used for the first ethanol fractionation as described by Ochoa. All centrifuging was done at 0°.

The total yield of enzyme was about 10% of that present initially. The specific activity of the final enzyme preparation was 6.5 times that of the supernatant from the dialysed solution. The assay was under the conditions shown in Fig. 1, which gave the maximum rate of reduction of oxidized triphosphopyridine nucleotide (TPN⁺). The final protein concentration was 3.4 mg./ml., as measured by the biuret method with bovine serum albumin (The Armour Laboratories) as standard. The enzyme solution was diluted tenfold as required and 0.1 ml. used in the cuvette. The protein concentration in the reaction mixture was thus 0.011 mg./ml.

The enzyme preparation contained no measurable fumarase or malic dehydrogenase activity, but some lactic dehydrogenase activity. This impurity, however, did not interfere with the assay of the malic enzyme activity, since only initial rates were measured and the pyruvate concentration was negligible.

Special chemicals. Two preparations of TPN⁺ were used (referred to as 'pure' and 'impure'). The pure preparation, obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., was approx. 95% pure. The impure preparation, prepared according to the method of Kornberg & Horecker (1953), contained 50% of TPN⁺ and 14% of diphosphopyridine nucleotide. The molar extinction coefficient of reduced triphosphopyridine nucleotide (TPNH) at 340 mμ

was taken as 6220 (Kornberg & Horecker, 1953). Dr P. W. Kent kindly supplied 2-fluorocitrate as its triethyl ester; this was hydrolysed by heating with the theoretical amount of NaOH at 100° for 1 hr.

Measurement of enzyme activity. The reaction was followed by measuring the increase in absorption at 340 mμ, this being assumed to represent reduction of TPN⁺. The reaction was carried out in 1 cm. silica cells in a Beckman spectrophotometer (model DU). The temperature of the cell was maintained at 30° by means of a thermostat cell-holder. Under the test conditions pyruvate had a molar extinction coefficient of 17 and malate of about 2. Change in absorption due to these compounds was ignored.

The reaction medium, which had a total volume of 3 ml., contained 0.1 ml. of enzyme, 0.3 μmole of TPN⁺ and varying amounts of 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer, malate, activators and inhibitors. Details are given with the individual experiments. The pH of the buffer given is that at 30°. L-Malate was used unless otherwise stated.

The reactants were added to the cell while it was in position in the cell-holder. All the reactants were at 30° before addition except for the enzyme and TPN⁺, which were kept at 0°. After the addition of all the reactants except enzyme, 2 min. was allowed for temperature equilibration. During this equilibration period a zero-time reading was taken. The enzyme was added to start the reaction and the cell contents were stirred vigorously for 10–15 sec. The first reading was taken 30 sec. after the addition of the enzyme and subsequent readings were taken every 30 sec. for 4 min., or every 15 sec. for 2 min., depending on the speed of the reaction. No difference was found when the reaction was started by addition of TPN⁺.

The initial rate was constant over at least five readings; all rates mentioned in the Results section are these initial rates.

Analysis of kinetics. The dissociation constants of enzyme-inhibitor complexes were obtained by the method of Dixon (1953). The terms $1/v$, $1/[S]$ and $[I]$ refer respectively to the reciprocal of the initial velocity, the reciprocal of the substrate concentration and to the inhibitor concentration.

RESULTS

Effect of malate concentration. The rate of TPN⁺ reduction increased with the malate concentration until the latter reached about mM, and then fell. The shape of the curve varied with the Mn²⁺ ion concentration (Fig. 1). The peak of the curve was at a higher malate concentration when the MnCl₂ concentration was greater. Similar curves were obtained with Mg²⁺ ions as the cofactor, but the decrease in the rate at high malate concentration was not so great.

The inhibition of the reaction by a high concentration of malate was thought to be due to chelation of the bivalent cations by malate. To test this the Mn²⁺ ion concentration was increased, and as expected the rate at high malate concentration rose (Fig. 2). There was no inhibition by high malate concentration when the Mn²⁺ ion concentration was increased stoichiometrically with the malate.

The shape of the curves also depended on pH (Fig. 3). At pH 7.7 the peak in the curve was higher and at a greater malate concentration. From Fig. 3 it appears that at higher malate concentrations (10 mM) the optimum pH is greater; this has been found to be so with the malic

enzymes from cattle lens (van Heyningen & Pirie, 1953) and from pigeon liver (Rutter & Lardy, 1958).

Fig. 1. Effect of malate concentration on malate decarboxylation. Cuvettes contained tris buffer, pH 7.2, (20 mM), TPN⁺ (0.3 μmole), enzyme (0.1 ml.) and malate and MnCl₂ as indicated, in a total volume of 3 ml. Δ, 0.33 mM-MnCl₂; ○, 3.3 mM-MnCl₂.

Fig. 2. Effect of increasing malate and MnCl₂ concentrations stoichiometrically on malate decarboxylation. Cuvettes contained tris buffer, pH 7.4 (0.1 M), impure TPN⁺ (0.3 μmole), enzyme (0.1 ml.) and malate and MnCl₂ as indicated, in a total volume of 3 ml.

Fig. 3. Effect of pH on malate decarboxylation. Cuvettes contained tris buffer (0.02 M), MgCl₂ (3.3 mM), impure TPN⁺ (0.3 μmole), enzyme (0.1 ml.) and malate as shown above, in a total volume of 3 ml.

Fig. 4. Effect of metal salts on malate decarboxylation. Cuvettes contained tris buffer, pH 7.2 (0.02 M), malate (mM), impure TPN⁺ (0.3 μmole), enzyme (0.1 ml.) and metal salts as indicated, in a total volume of 3 ml. Curve 1, CuSO₄; curve 2, ZnSO₄; curve 3, NiSO₄; curve 4, CoCl₂; curve 5, MgCl₂; curve 6, MnCl₂. The initial rate in the absence of added metal salt was 6 μm-moles of TPNH/min.

Table 1. *Effect of C₃-dicarboxylic acids on malate decarboxylation*

Each cuvette contained tris buffer, pH 7.4 (0.1 M), pure TPN⁺ (0.3 μ mole) and enzyme (0.1 ml.) in a total volume of 3 ml. Malate, inhibitor and metal salt concentrations were as given below. With mesoxalate the cation was Mg²⁺, with the others Mn²⁺.

Concn. of malate (mm)	Concn. of MnCl ₂ (mm)	Inhibition (%)				
		Malonate			Tartronate	
		20 mm	5 mm	2 mm	0.06 mm	0.03 mm
1.0	3.3	84	48	19	—	—
0.3	3.3	91	74	48	—	—
0.1	3.3	100	88	71	30	15
0.06	3.3	—	—	—	30	13
0.03	3.3	—	—	—	33	17
1.0	3.3	—	50	27	—	—
1.0	0.33	—	52	26	—	—
	Concn. of MgCl ₂ (mm)	Mesoxalate				
		mm	0.8 mm	0.5 mm	0.2 mm	
1.0	3.3	60	50	40	15	
0.6	3.3	—	63	52	23	
0.3	3.3	—	74	62	43	
1.0	0.03	69	—	—	—	

Effect of cations. As already mentioned (Fig. 1), the initial rate of malate decarboxylation was greatly influenced by the Mn²⁺ ion concentration and further data are given in Fig. 4. Mg²⁺ and Co²⁺ ions have also been reported to activate malic enzyme (Salles & Ochoa, 1950; Conn, Vennesland & Kraemar, 1949). In the present experiments these ions, and also Ni²⁺ ions, were found to activate (Fig. 4). The optimum concentration was between 3 mm and 10 mm for all four ions. Concentrations over 10 mm caused some inhibition. Highest initial rates were obtained with Mn²⁺ ions. The effects of the other cations decreased in the order Mg²⁺, Co²⁺ and Ni²⁺. Zinc sulphate inhibited the enzyme at concentrations greater than 0.3 mm; this inhibition was not due to the SO₄²⁻ ion since MnSO₄ activated to the same extent as MnCl₂. Copper sulphate was an even stronger inhibitor, complete inhibition being caused by a concentration of 0.3 mm.

Zinc sulphate (0.3 mm) inhibited 70 % in the presence of mm-Mn²⁺ ion, compared with 79 % with 0.3 mm-Mn²⁺ ion.

Calcium chloride had little effect on the rate of reaction when added together with MnCl₂. With 0.33 mm-MnCl₂, 3.3 mm-CaCl₂ increased the initial rate from 52 to 56 μ m-moles of TPNH/min. Calcium chloride (20 mm) inhibited approximately 90 % when added alone.

The univalent ions, K⁺, Na⁺, Li⁺ and NH₄⁺, at a concentration of 0.02 M, had no effect on the reaction rate.

Effect of dicarboxylic acids. Malonate, tartronate and mesoxalate all inhibit the decarboxylation of malate (Table 1).

Fig. 5. Effect of malonate and mesoxalate on malate decarboxylation. Top graphs are for malonate, bottom graphs are for mesoxalate. Left-hand graphs are plots of $1/v$ against $1/[S]$; the figures on the graph indicate the inhibitor concentration. Right-hand graphs are plots of $1/v$ against $[I]$; the figures on the graphs indicate the substrate concentration. Tris buffer, TPN⁺ and enzyme were as given in Table 1. With malonate, 3.3 mm-MnCl₂ was present, and with mesoxalate, 3.3 mm-MgCl₂. (v , Initial velocity as μ m-moles of TPNH formed in 1 min. $[S]$ and $[I]$ are substrate and inhibitor concentrations.)

Malonate is a mainly competitive inhibitor; in a $1/v$ against $1/[S]$ plot the lines cross almost on the ordinate (Fig. 5). Decreasing the malate concentration from mm to 0.1 mm increased the inhibition by 2 mm-malonate from 19 to 71 % (Table 1). A ten-fold decrease in MnCl₂ concentration did not alter the inhibition (27 % at 3.3 mm and 26 % at 0.3 mm-

MnCl_2), showing that malonate did not inhibit by chelating cations. Mesoxalate is also a competitive inhibitor (Fig. 5). Since mesoxalate forms an insoluble compound with MnCl_2 , MgCl_2 was used as activator for the enzyme. Changing the malate concentration from mM to 0.3 mM at 0.5 mM-mesoxalate brought about an increase of inhibition from 40 to 62%. With both malate and mesoxalate at mM, decrease in the MgCl_2 concentration from 3.3 mM to 0.033 mM only increased the inhibition from 60 to 69%, which suggests that mesoxalate did not inhibit by binding cations.

Fig. 6. Effect of tartronate on malate decarboxylation. Upper graphs show the effect at higher concentrations and the bottom graphs that at lower concentrations of tartronate. MnCl_2 concentration was 3.3 mM. Other details are as given in Fig. 5.

Table 2. Values of K_i for various inhibitors of malic enzyme

Values of K_i were obtained graphically by the method of Dixon (1953).

Inhibitor	K_i (mM)
Malonate	0.6
Tartronate	0.1
Mesoxalate	0.1
Succinate	20
L-Aspartate	10
mesoTartrate	2

The figures in Table 1 indicate that tartronate is a non-competitive inhibitor, the increase in inhibition by 0.06 mM-tartronate being only from 30 to 33% when the malate was decreased from 0.1 mM to 0.03 mM. At high tartronate concentrations (mM), both $1/v$ against $1/[S]$ and $1/v$ against $[I]$ plots were non-linear (Fig. 6). However, at concentrations of 0.1 mM and below these plots gave straight lines which crossed in the upper left quadrant of the graph, showing the inhibition must have some competitive element (Fig. 6). A part of the tartronate inhibition was due to the chelation of cations, as a tenfold decrease in MnCl_2 concentration (3.3 mM to 0.33 mM) increased the inhibition from 52 to 77% in the presence of mM-malate and 0.3 mM-tartronate. The fact that tartronate appears to inhibit in two ways (by competition with malate and by chelation with Mn^{2+} ions) may explain the non-linearity of the $1/v$ against $1/[S]$ graph at high tartronate concentrations.

All three of the C_3 -dicarboxylic acids gave K_i values of between 0.1 mM and 0.6 mM (see Table 2).

A number of C_4 -dicarboxylic acids also inhibited malate decarboxylation. Succinate and L-aspartate inhibited to an equal extent (Table 3). With inhibitor concentrations of 20 mM, decrease in the malate concentration from 0.1 mM to 0.03 mM increased the inhibition from 46 to 52% for succinate and from 52 to 57% for aspartate. As for tartronate, the figures in Table 3 suggest that these inhibitors are largely non-competitive, but for succinate a $1/v$ against $1/[S]$ plot (in which the lines crossed in the upper left quadrant) showed the inhibition to be partially competitive (Fig. 7). The graph for aspartate was non-linear (Fig. 7). At the same inhibitor concentration as above (20 mM) and 0.1 mM-malate, decreasing the MnCl_2 concentration from 3.3 mM to 0.33 mM changed the inhibition due to succinate from 46 to 56% and that due to aspartate from 52 to 50%. The K_i values for these two inhibitors were about 10 mM (Table 2).

DL-Malate gave the same initial rate as half its concentration of L-malate. D-Malate thus had no effect when present in a concentration equal to that of L-malate.

mesoTartrate had an inhibitory power approximately midway between those of succinate and the

Table 3. Effect of succinate and aspartate on malate decarboxylation

Cuvette contents were as in Table 1, except for MnCl_2 , malate and inhibitor, which were as given below.

Concn. of malate (mM)	Concn. of MnCl_2 (mM)	Inhibition (%)					
		Succinate			L-Aspartate		
		30 mM	20 mM	10 mM	20 mM	10 mM	4 mM
0.1	3.3	54	46	31	52	32	12
0.06	3.3	56	44	28	52	36	14
0.03	3.3	57	52	24	57	48	9
0.1	0.33	—	56	—	50	—	—

C₃-acids (Table 4); its value for *K_i* was about mm. The inhibition by *mesotartar*ate was competitively reversed by malate (Fig. 8 and Table 4). With *mm-mesotartar*ate there was 33 % inhibition with 0.02 mm-malate but only 4 % inhibition with

0.1 mm-malate. To some extent its effect was due to chelation of cations: the 4 % inhibition quoted above was increased to 28 % if the Mn²⁺ ion concentration was decreased 50-fold. This increase was not so great (48–59 %) with 3 mm-*mesotartar*ate.

Inhibition by oxaloacetate was much more powerful. Marked inhibition was obtained with oxaloacetate concentrations less than the malate concentration (Table 5). An oxaloacetate concentration of 0.167 mm inhibited 66 % with mm-malate and 91 % with 0.1 mm-malate. Decreasing the MnCl₂ concentration to one-tenth (to 0.33 mm) had no effect on the inhibition (see Table 5). The inhibition due to oxaloacetate was thus competitive and did not involve the chelation of cations. In these experiments, Mn²⁺ ions were present together with the oxaloacetate, and it is known that oxaloacetate can be decarboxylated non-enzymically in the presence of Mn²⁺ ions (Krebs, 1942). The extinction at 290 mμ was used as a measure of the oxaloacetate concentration in the solution since, at this wavelength, oxaloacetate absorbs, but TPNH has very little absorption. During the time of the temperature equilibration and the reaction, the decrease in the oxaloacetate concentration was always less than 10 %.

As shown in Table 6, *D*-tartrate inhibited to about the same extent as *mesotartar*ate. With 0.1 mm-malate, *D*-tartrate concentrations of 2 mm inhibited 56 % and of 0.6 mm, 25 %. Inhibition by fumarate and maleate was relatively weak. With the inhibitor concentration (10 mm) 100 times the malate concentration (0.1 mm), fumarate inhibited

Fig. 7. Effect of succinate and L-aspartate on malate decarboxylation. Upper graphs refer to succinate and the lower ones to L-aspartate. Other details are as given in Fig. 5 (3.3 mm-MnCl₂ was present).

Fig. 8. Effect of *mesotartar*ate on malate decarboxylation. Plots of 1/*v* against 1/[*S*] (on which the figures indicate the inhibitor concentration) and of 1/*v* against [*I*] (the figures giving the substrate concentration) for *mesotartar*ate. Tris buffer, TPN⁺ and enzyme were as given in Table 1. The MnCl₂ concentration was 3.3 mm. *v*, Initial velocity (μmoles of TPNH formed in 3 min.).

Table 4. Effect of *mesotartar*ate on malate decarboxylation

Cuvette contents were as in Table 1, except for MnCl₂, malate and *mesotartar*ate, which were as given below.

Concn. of malate (mm)	Concn. of MnCl ₂ (mm)	Inhibition (%) by <i>mesotartar</i> ate		
		10 mm	3 mm	mm
0.1	3.3	77	48	4
0.05	3.3	83	61	41
0.02	3.3	100	60	33
0.1	0.066	—	59	28

Table 5. Effect of oxaloacetate on malate decarboxylation

Cuvette contents were as in Table 1, except for MnCl₂, malate and oxaloacetate, which were as given below.

Concn. of malate (mm)	Concn. of MnCl ₂ (mm)	Inhibition (%) by oxaloacetate	
		0.167 mm	0.067 mm
1	3.3	66	49
0.1	3.3	91	79
1	0.33	64	—

Table 6. *Effect of other C₄-dicarboxylic acids on malate decarboxylation*

Cuvette contents were as in Table 1, except for MnCl_2 , malate and inhibitors, which were as given below.

Concn. of malate (mm)	Concn. of MnCl_2 (mm)	Inhibition (%)				
		D-Tartrate			Fumarate (10 mm)	Maleate (10 mm)
		10 mm	2 mm	0.6 mm		
1	3.3	65	24	—	8	8
0.1	3.3	—	56	25	31	21
0.1	0.033	—	—	29	53	28

Table 7. *Effect of oxalate on malate decarboxylation*

Cuvettes contained tris buffer, pH 7.2 (0.02M), MnCl_2 (0.02M), impure TPN^+ (0.3 μ mole) and enzyme (0.1 ml.) in a total volume of 3 ml. Malate and oxalate were as given below.

Concn. of malate (mm)	Inhibition (%) by oxalate		
	3 mm	2 mm	0.75 mm
10	72	63	—
1	77	—	56

Table 8. *Effect of citrate on malate decarboxylation*

MnCl_2 , malate and citrate were as given below; other contents were as given in Table 1.

Concn. of malate (mm)	Concn. of MnCl_2 (mm)	Inhibition (%) by citrate	
		20 mm	10 mm
1	3.3	28	25
0.1	3.3	23	6
0.1	0.033	95	—

31 % and maleate 21 %. These two figures were increased to 53 and 28 % respectively when the MnCl_2 concentration was reduced from 3.3 mm to 0.033 mm.

Oxalate proved a strong inhibitor of the enzyme (Table 7). At 3 mm-oxalate, there was 72 % inhibition in the presence of 10 mm-malate and 77 % inhibition in the presence of mm-malate.

Effect of other carboxylic acids on malate decarboxylation. Citrate (20 mm) inhibited 23 % at a MnCl_2 concentration of 3.3 mm and with 0.1 mm-malate (Table 8). At a MnCl_2 concentration only 1 % of this, there was almost complete inhibition (95 %), indicating that a large part of the inhibition was due to the binding of Mn^{2+} ions.

Citrate required concentrations in excess of 10 mm for appreciable inhibition. Fluorocitrate, however, inhibited to the same extent at concentrations of mm (Table 9). Thus with mm-fluorocitrate there was 35 % inhibition with 10 mm-malate and 76 % inhibition with mm-malate (the MnCl_2 concentration was 0.33 mm). The inhibition was competitive, but, as with citrate, some of the inhibition also appeared to be due to chelation with Mn^{2+} ions. With both fluorocitrate and malate at a concentra-

tion of mm, decrease of the MnCl_2 concentration from 17 mm to 0.33 mm increased the inhibition from 34 to 76 %.

Four C₃-monocarboxylic acids were studied: propionate, D- and L-lactate and pyruvate. At a concentration of 10 mm in the presence of mm-malate, propionate did not inhibit, D- and L-lactate caused identical inhibition (23 %) and pyruvate inhibited 16 % (Table 10). A tenfold increase in either the malate or the MnCl_2 concentration had almost no effect on the inhibition by any of these acids.

DL- β -Hydroxybutyrate (10 mm) caused no inhibition and DL- α -hydroxybutyrate (10 mm) inhibited 19 % under the conditions given above for lactate inhibition (see Table 10).

DISCUSSION

About 50 % inhibition of the initial rate of conversion of malate into pyruvate was obtained with oxaloacetate at 0.01 mm, malonate, tartronate and mesoxalate at 0.3 mm, oxalate and fluorocitrate at about mm, mesotartarate at 3 mm, D-tartrate and fumarate at 10 mm and succinate and L-aspartate at 20 mm. Maleate, citrate, D- and L-lactate, pyruvate and DL- α -hydroxybutyrate inhibited about 20 % at a concentration of 10 mm. Under the same conditions propionate and DL- β -hydroxybutyrate did not inhibit. These data apply to an L-malate concentration of 0.1 mm, except for oxalate and fluorocitrate, which refer to mm-L-malate.

The inhibitions of the malic enzyme appear to consist of two different elements: the chelation of the bivalent cations (Mn^{2+} and Mg^{2+}), which act as cofactors for the enzyme, and a true inhibition of the enzyme. That chelation of ions plays a role is suggested by the fact that the inhibition can be partly overcome by the addition of cations. This applies especially to the inhibition by citrate, by high concentrations of malate and to a less extent to the other inhibitory substances, as judged by the reduction of the inhibition on addition of Mn^{2+} or Mg^{2+} ions. The analysis of the inhibitions by the methods indicated by Dixon (1953) shows that the inhibitions were partly competitive and partly

Table 9. *Effect of fluorocitrate on malate decarboxylation*

Cuvettes contained tris buffer (pH 7.2, 0.02M in Expt. 1; pH 7.4, 0.1M in Expt. 2), pure TPN⁺ (0.3 μ mole) and enzyme (0.1 ml.) in a total volume of 3 ml. Malate, MnCl₂ and fluorocitrate were as given below.

Expt. no.	Concn. of malate (mM)	Concn. of MnCl ₂ (mM)	Inhibition (%) by fluorocitrate				
			10 mM	4 mM	mM	0.1 mM	0.01 mM
1	10	17	—	—	27	—	—
	10	0.33	—	—	35	—	—
	1	17	—	—	34	—	—
	1	0.33	—	—	76	—	—
2	1	3.3	84	—	36	14	2
	1	0.33	—	84	59	14	0

Table 10. *Effect of monocarboxylic acids on malate decarboxylation*

Cuvette contents were as given in Table 1, except for MnCl₂ and malate, which were as given below. Concentration of the monocarboxylic acids was 10 mM.

Concn. of malate (mM)	Concn. of MnCl ₂ (mM)	Inhibition (%)					
		Propionate	D-Lactate	L-Lactate	Pyruvate	DL- α -Hydroxy-butyrate	DL- β -Hydroxy-butyrate
1	3.3	0	23	23	16	19	0
0.1	3.3	0	22	22	18	21	0
0.1	0.33	0	23	23	19	—	—
0.1	0.033	—	—	—	—	28	0

non-competitive, which could be explained if the two different elements were responsible for the inhibition. The competitive element is not unexpected since all the effective inhibitors resemble malate structurally in that they possess either two carboxyl groups, or one carboxyl plus an α -hydroxyl or carbonyl group. These substances presumably compete with the malate for the binding sites of the enzyme.

Gal (1958) found very little inhibition of the malic enzyme by fluorocitrate, but the highest concentration tried by him was 0.066 mM. He found a 60% inhibition by 0.7 mM-fluoromalate. The only other carboxylic acids which have previously been shown to inhibit malate decarboxylation are malonate and fumarate (van Heyningen & Pirie, 1953). The same authors showed that impure sodium ethyl oxaloacetate was inhibitory.

The exact mechanism of the malic enzyme reaction is still unknown, but an oxaloacetate-enzyme complex is the most probable intermediate (see Rutter & Lardy, 1958). In this connexion it is perhaps significant that oxaloacetate was by far the most powerful inhibitor of malic enzyme found. The decarboxylation of oxaloacetate by malic enzyme is known to be inhibited by malonate, D- and L-malate, succinate and fumarate (Salles & Ochoa, 1950), but whether the same site on the enzyme is involved in this reaction is not known.

The activation of the enzyme by cations was greatest by Mn²⁺ ions, Mg²⁺, Co²⁺ and Ni²⁺ ions being less effective; Cu²⁺ and Zn²⁺ were inhibitory.

In the experiments of Rutter & Lardy (1958) the most active ion was Co²⁺. They obtained slight inactivation by Zn²⁺ and Cu²⁺ ions. These differences might be due to the greater purity of the enzymes used by Rutter & Lardy, or to the presence of cysteine in their assay medium.

SUMMARY

1. Some aspects of the kinetics, and of the inhibition by carboxylic acids, of the conversion of L-malate into pyruvate have been investigated.

2. Mn²⁺ ions greatly increased the rate of malate decarboxylation. Mg²⁺, Co²⁺ and Ni²⁺ ions also activated the enzyme, but the maximum rate obtained was less than with Mn²⁺ ions. Cu²⁺, Zn²⁺ and Ca²⁺ ions inhibited the enzyme.

3. The decarboxylation of malate was inhibited by high malate concentrations. Since the inhibition was reversed by increased Mn²⁺ ion concentrations, it was probably due to chelation of Mn²⁺ ions by the malate.

4. All the dicarboxylic acids tested inhibited the reaction. The inhibitory power decreased in the following order: oxaloacetic acid (which inhibited at concentrations less than that of the substrate), tartronic acid, mesoxalic acid, malonic acid, oxalic acid, mesotartaric acid, D-tartaric acid, L-aspartic acid, succinic acid, fumaric acid and maleic acid. For the last four acids concentrations 100 times that of the substrate were required for appreciable inhibition.

5. D- and L-Lactate, pyruvate and DL- α -hydroxybutyrate inhibited slightly at high concentrations. Propionate and DL- β -hydroxybutyrate were not inhibitory at the same concentrations.

6. Citrate and fluorocitrate also inhibited malate decarboxylation. For equal inhibition, the concentration of citrate required was over ten times that of the fluorocitrate.

7. The inhibition by di- and tri-carboxylic acids was partly reversed by addition of Mn^{2+} (or Mg^{2+}) ions and partly by raising the substrate concentration. The data are in accordance with the view that the inhibitions are due partly to chelation of the bivalent cations required as cofactors and partly to competition with the malate for the enzyme-binding site.

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Some Properties of the Malic Enzyme of Pigeon Liver

2. SYNTHESIS OF MALATE FROM PYRUVATE

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The kinetics of the decarboxylation of malate by the malic enzyme of pigeon liver and of the inhibition of the reaction by carboxylic acids was the subject of the preceding paper (Stickland, 1959). In this paper are reported the results of a similar study on the synthesis of malate by the reaction:

where TPNH and TPN^+ stand for reduced and oxidized triphosphopyridine nucleotide respectively.

EXPERIMENTAL

Enzyme preparation. The enzyme preparation was the same as that used previously (Stickland, 1959). The preparation contained considerable lactic dehydrogenase activity. The rates observed were therefore corrected for

the rate of oxidation of reduced triphosphopyridine nucleotide (TPNH) by pyruvate in the absence of added bicarbonate. Under these conditions the malic enzyme activity was not completely abolished since the solutions were not completely free from CO_2 . Thus the corrections represented the maximum value for the activity of lactic dehydrogenase.

Special chemicals. Oxidized triphosphopyridine nucleotide (TPN^+) and TPNH (enzymically reduced) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Another sample of TPNH was prepared from TPN^+ by reduction with dithionite (Beisenherz *et al.* 1953). As indicated by absorption at 340 $m\mu$ the commercial preparation contained about 73% of TPNH by weight and the sample prepared chemically contained TPNH equal to about 80% of the TPN^+ added. The molar extinction coefficient of TPNH was taken as 6220 (Kornberg & Horecker, 1953).

The fluorocitrate preparation used was described previously (Stickland, 1959).

Measurement of enzyme activity. The reaction was followed as a decrease in absorption at 340 $m\mu$ (this being assumed to represent oxidation of TPNH) and was carried out in the apparatus used for the study of malate decarboxylation

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