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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(Stickland, 1959). Under the test conditions pyruvate had a molar extinction coefficient at 340 $m\mu$ of 17. As a result pyruvate concentrations greater than 50 mM could not be used, as the absorption was too high. Changes in absorption due to change in the pyruvate concentration were ignored.

The reaction medium, which had a total volume of 3 ml., contained 0.1 ml. of enzyme, 0.2 ml. of TPNH (0.15 μ mole), 0.1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.4 at 30°, and varying amounts of pyruvate, bicarbonate, activators and inhibitors. Details are given with the individual experiments.

The reactants were added while the cell was in the cell-holder. All reactants were at 30° before addition, except for the enzyme and TPNH, which were kept at 0°. A $\text{CO}_2 + \text{N}_2$ gas mixture was bubbled through the cell during the whole of the equilibration period and during the reaction. The bicarbonate concentration in the cell and the percentage of CO_2 in the gas phase were adjusted to give pH 7.4. The required concentrations were read from the graph given by Umbreit (1949). After temperature equilibration for at least 2 min. the TPNH was added and readings were taken to observe any non-enzymic rate of TPNH removal. Enzyme was added 3 min. after the addition of TPNH and readings were taken every 30 sec. for 4 or 5 min. The initial rate was constant over at least five readings; all rates mentioned in the Results section are these initial rates.

Analysis of kinetics. Apparent Michaelis constants obtained under one set of conditions only were determined graphically by the method of Lineweaver & Burk (1934). The type of inhibition was established by the graphical method of Dixon (1953).

RESULTS

Non-enzymic removal of TPNH. Preliminary studies on the oxidation of TPNH by pyruvate and bicarbonate- CO_2 mixtures showed that chemically reduced TPNH disappeared in the absence of enzyme, particularly in the presence of high concentrations of MnCl_2 or bicarbonate. This removal was catalysed readily by Mn^{2+} , Co^{2+} , Ni^{2+} and Cu^{2+} ions, but only to a small extent by Mg^{2+} , Zn^{2+} and Ca^{2+} ions (Table 1). The rate of removal in the presence of Mn^{2+} , Co^{2+} and Ni^{2+} ions was increased by bicarbonate (from 3.5 to 40 μ m-moles of TPNH/3 min. for 3.3 mM- Mn^{2+} ions). Cu^{2+} ions were much more effective: in the absence of bicarbonate 0.33 mM- Cu^{2+} ions gave an initial rate of 43 μ m-moles/3 min., whereas ten times this concentration of Mn^{2+} ions gave a rate of only 2 μ m-moles/3 min. (Table 1, Expt. 3). However, bicarbonate decreased the rate in the presence of 0.33 mM- Cu^{2+} ions from 43 to 36 μ m-moles of TPNH/3 min. In the presence of bicarbonate the rates with 0.33 mM- Cu^{2+} ions and 3.3 mM- Mn^{2+} ions were not very different, being 36 and 25 μ m-moles/3 min. respectively.

Rackis & Kalnitsky (1957) found a non-enzymic oxidation of α -oxoglutarate by Mn^{2+} and Co^{2+} ions, which was stimulated by amino acids, particularly threonine. Threonine increased the rate of non-enzymic removal of TPNH in the presence of

Table 1. *Non-enzymic oxidation of reduced triphosphopyridine nucleotide*

Cuvettes contained tris buffer, pH 7.4 (0.1 M), TPNH (0.15 μ mole) and metal salts, bicarbonate and threonine as indicated. In Expts. 1-3 chemically reduced TPNH was used; in Expt. 4 enzymically reduced TPNH was used. NaHCO_3 was 0.067 M; when NaHCO_3 was added $\text{CO}_2 + \text{N}_2$ (20:80) was bubbled through cuvettes (pH 7.4). Threonine was 3.3 mM.

Expt. no.	Additions	Initial rate of TPNH oxidation (μ m-moles/3 min.)		
		No NaHCO_3 or threonine	+ NaHCO_3	+ Threonine
1	None	0	1	—
	MnCl_2 (3.3 mM)	3.5	40	—
	CaCl_2 (3.3 mM)	1	3.5	—
	MgCl_2 (3.3 mM)	1	1.5	—
	ZnSO_4 (3.3 mM)	0	2.5	—
2	MnCl_2 (3.3 mM)	4	33	—
	CoCl_2 (0.33 mM)	7	16	—
	NiSO_4 (0.33 mM)	6	23	—
3	None	0	2.5	2
	MnCl_2 (3.3 mM)	2	25	10
	CuSO_4 (0.33 mM)	43	36	6
4	None	0	0	0
	MnCl_2 (3.3 mM)	0	0	0
	CuSO_4 (0.33 mM)	1	0	—
	'Oxidized dithioniate' (0.4 mM)	0	2	—
	'Oxidized dithioniate' (0.4 mM) + MnCl_2 (3.3 mM)	2	19	—
	Na_2SO_3 (0.5 mM)	1	2	—
	Na_2SO_3 (0.5 mM) + MnCl_2 (3.3 mM)	6	60	—
	Na_2SO_3 (0.5 mM) + CuSO_4 (0.33 mM)	64	11	—

Mn^{2+} ions from 2 to 10 $\mu\text{m-moles/3 min.}$; that in the presence of Cu^{2+} ions was decreased from 43 to 6 $\mu\text{m-moles/3 min.}$

Mn^{2+} and Cu^{2+} ions caused almost no removal of enzymically reduced TPNH (Table 1, Expt. 4). During the chemical reduction of TPN^+ , the dithionite used is oxidized mainly to sulphite. Assuming all the dithionite was oxidized to sulphite, the final concentration of sulphite in the cell was approximately 0.4 mM. The removal of enzymically reduced TPNH plus either 0.4 mM-oxidized dithionite (dithionite treated in the same manner as in TPN^+ reduction, but in the absence of TPN^+) or 0.5 mM-sulphite had the same characteristics as that of chemically reduced TPNH. The rate with Mn^{2+} ions was increased by bicarbonate (6–60 $\mu\text{m-moles/3 min.}$) and the rate with Cu^{2+} ions was decreased by bicarbonate (64–11 $\mu\text{m-moles/3 min.}$) (Table 1, Expt. 4). Thus the above-mentioned effects of the cations on chemically reduced TPNH were presumably dependent on the presence of sulphite. In all subsequent experiments enzymically reduced TPNH was used.

Effect of substrate concentration. The slow rate of TPNH oxidation in the absence of added bicarbonate was rapidly raised when the bicarbonate- CO_2 concentration was increased, a maximum being reached at about 0.15M- NaHCO_3 (45% of CO_2) (Fig. 1). Higher bicarbonate concentrations caused some inhibition.

Fig. 1. Effect of NaHCO_3 and CO_2 on malate synthesis. Cells contained tris buffer, pH 7.4 (0.1M), MnCl_2 (0.33 mM), TPNH (0.15 μmole) and enzyme (0.1 ml.) in a total volume of 3 ml. CO_2 was in the gas phase as shown above. Concentration of NaHCO_3 was varied to keep the pH at 7.4. ●, No pyruvate; Δ, 10 mM-pyruvate; ○, 50 mM-pyruvate. There was no precipitation of Mn^{2+} ions at the higher bicarbonate concentration.

Fig. 2. Effect of pyruvate concentration on malate synthesis. ●, No CO_2 or NaHCO_3 ; ○, 20% of CO_2 and 66 mM- NaHCO_3 ; Δ, 60% of CO_2 and 0.198M- NaHCO_3 . Other conditions were as given in Fig. 1. In the absence of pyruvate the initial rate was approx. 0.5 $\mu\text{m-mole}$ of $\text{TPN}^+/\text{3 min.}$

Fig. 3. Effect of metal salts on malate synthesis. Cells contained tris buffer, pH 7.4 (0.1M), pyruvate (10 mM), NaHCO_3 (66 mM), TPNH (0.15 μmole), enzyme (0.1 ml.) and metal salts as shown, in a total volume of 3 ml. ●, MnCl_2 ; ○, MgCl_2 ; □, CoCl_2 ; ▲, CuSO_4 ; Δ, ZnSO_4 . CuSO_4 and ZnSO_4 curves were obtained in the presence of 0.033 mM- MnCl_2 . The initial rate in the absence of added metal salt was approx. 10 $\mu\text{m-moles}$ of $\text{TPN}^+/\text{3 min.}$

Table 2. *Effect of C₃-dicarboxylic acids on malate synthesis*

Cuvettes contained tris buffer, pH 7.4 (0.1M), NaHCO₃ (0.2M), TPNH (0.15 μ mole), enzyme (0.1 ml.) and pyruvate, metal salts and inhibitors as given below, in a total volume of 3 ml. Gas phase, CO₂ + N₂ (60:40). With mesoxalate Mg²⁺ ions were used as activator; with the other inhibitors Mn²⁺ ions were used.

Concn. of pyruvate (mm)	Concn. of MnCl ₂ (mm)	Inhibition (%)			
		Malonate		Tartronate	
		10 mm	mm	0.1 mm	0.01 mm
50	0.33	46	10	70	24
10	0.33	70	37	74	32
1	0.33	62	43	—	—
50	0.0033	—	—	43	3
	Concn. of MgCl ₂ (mm)	Mesoxalate			
		20 mm	5 mm	mm	
50	3.3	65	45	30	
10	3.3	43	36	12	
50	0.033	—	50	21	

In the absence of pyruvate there was no TPNH oxidation (Fig. 2). Values for apparent K_m of pyruvate and CO₂ were 2×10^{-2} M and 2×10^{-3} M respectively. The figure for CO₂ was calculated from the percentage of CO₂ in the gas phase required to give half the maximum rate.

Requirement for bivalent cations. The presence of bivalent cations was necessary for the maximum rate of malate synthesis. On increasing the MnCl₂ concentration a maximum rate was reached at approximately 0.1 mm (Fig. 3). Higher MnCl₂ concentrations (up to 3.3 mm) did not cause any inhibition. Mg²⁺ and Co²⁺ ions also activated the enzyme. At 0.033 mm they had little effect, but on increasing their concentrations the rate of reaction rose. A maximum rate was not reached by a concentration of 3.3 mm. Nickel sulphate activated the enzyme to the same extent as CoCl₂ at a concentration of 0.33 mm.

Copper sulphate and ZnSO₄ both inhibited when added together with 0.033 mm-MnCl₂. At a concentration of 2 mm, CuSO₄ and ZnSO₄ decreased the initial rate from 31 to 15 and 4 μ m-moles/3 min. respectively.

Effect of carboxylic acids. Malonate, tartronate and mesoxalate inhibited the synthesis of malate. The degree of inhibition depended in some cases on the substrate concentration. Thus on decreasing the pyruvate concentration from 50 mm to 10 mm, the inhibition by mm-malonate was increased from 10 to 37 %, that by 0.1 mm-tartronate remained about the same (70–74 %) and that by 20 mm-mesoxalate was decreased from 65 to 43 % (Table 2). These figures suggest that the inhibition by malonate was competitive and that that by tartronate had a large non-competitive element. The tartronate inhibition was decreased by lowering the MnCl₂ concentration: with 50 mm-pyruvate and 0.1 mm-tartronate, the

Fig. 4. Effect of tartronate and mesoxalate. Plots of reciprocal of velocity against mesoxalate and tartronate concentrations. The figures on the graphs indicate the pyruvate concentration. v , Initial velocity as μ mole of TPN⁺ formed/3 min.

inhibition was 70 % at 0.33 mm-MnCl₂ and 43 % at 3.3 μ M-MnCl₂. Since the mesoxalate inhibition decreases with decreasing substrate concentration, it appears that the inhibition is uncompetitive (see Ebersole, Guttentag & Wilson, 1944). However, in all cases plots of the reciprocal of the velocity ($1/v$) against inhibitor concentration and of $1/v$ against the reciprocal of the substrate concentration were non-linear (see Fig. 4), showing that first-order kinetic theory does not apply.

Three C₄-dicarboxylic acids (succinic acid, meso-tartaric acid and oxaloacetic acid) were competitive in as much as the inhibition depended on the substrate concentration. Decrease in the pyruvate concentration from 50 to 10 mm resulted in increased inhibition by 20 mm-succinate (31–47 %), 10 mm-mesotartaric (14–24 %) and 10 mm-oxaloacetate (47–62 %) (Table 3).

Inhibition by D- and L-lactate and DL- α -hydroxybutyrate also depended on the pyruvate concentration (Table 4). Thus with two pyruvate

Table 3. *Effect of C₄-dicarboxylic acids on malate synthesis*

Cuvette contents were as given in Table 2; MnCl₂ concentration was 0.33 mM. Pyruvate and inhibitors were as given below.

Concn. of pyruvate (mM)	Inhibition (%)					
	Succinate		<i>meso</i> Tartrate		Oxaloacetate	
	50 mM	10 mM	10 mM	2 mM	10 mM	mM
50	31	0	14	—	47	6
10	47	11	24	2	62	26

Table 4. *Effect of monocarboxylic acids on malate synthesis*

Cuvette contents were as given in Table 3.

Conc. of pyruvate (mm)	Inhibition (%)						
	D-Lactate		L-Lactate (20 mm)	Propionate (20 mm)	DL- α -Hydroxy- butyrate		DL- β - Hydroxy- butyrate (20 mm)
	20 mm	5 mm			20 mm	5 mm	
50	23	9	23	0	26	6	—
10	50	13	47	0	39	17	2

Table 5. *Effect of citrate and fluorocitrate on malate synthesis*

Cuvette contents were as given in Table 2, except pyruvate, MnCl₂ and inhibitors, which were as given below.

Concn. of pyruvate (mM)	Concn. of MnCl ₂ (mM)	Inhibition (%)		
		Fluorocitrate		Citrate (20 mM)
		0.167 mM	0.0167 mM	
50	0.33	39	11	25
10	0.33	50	25	13
50	0.033	28	—	44

Table 6. *Comparison of the inhibitory effects of di- and tri-carboxylic acids on the decarboxylation and synthesis of malate*

Values for malate decarboxylation are from Stickland (1959) and were obtained in the presence of 0.1 mM-malate. For malate synthesis, 10 mM-pyruvate was used. These differing substrate concentrations were necessary in order to obtain measurable rates of reaction.

Inhibitor	Concn. of inhibitor (mM) required for 50% inhibition of the initial rate of	
	Malate decarboxylation	Malate synthesis
Oxaloacetate	0.01	10-1
Malonate	0.3	1
Tartronate	0.3	0.10-0.01
Mesoxalate	0.3	20
Oxalate	1.0	—
Fluorocitrate	1.0	0.1
<i>meso</i> Tartrate	3.0	> 10
D-Tartrate	10.0	—
Fumarate	10.0	—
Succinate	20.0	50
L-Aspartate	20.0	—

concentrations of 50 mM and 10 mM, 20 mM-D-lactate inhibited 23 and 50%, 20 mM-L-lactate 23 and 47% and 20 mM-DL- α -hydroxybutyrate 26 and 39% respectively. Propionate (20 mM) did not inhibit. At the lower of these pyruvate concentrations there was virtually no inhibition (2%) by 20 mM- β -hydroxybutyrate.

The inhibition by fluorocitrate and citrate depended on the substrate concentration, but that by 0.167 mM-fluorocitrate increased (39-50%) and that by 10 mM-citrate decreased (25-13%) when the pyruvate concentration was decreased from 50 to 10 mM (Table 5). A tenfold decrease in the MnCl₂ concentration (0.33-0.033 mM) lowered the inhibition by 0.167 mM-fluorocitrate from 39 to 28% but increased that by 10 mM-citrate from 25 to 44%.

DISCUSSION

The data show that the initial rate of the synthesis of malate by malic enzyme is inhibited by the same carboxylic acids as that of the decarboxylation of malate (see Table 6). However, the concentration required for 50% inhibition was, in most cases, different for the two directions of the reaction. Oxaloacetate was the only inhibitor of malate decarboxylation effective at a concentration less than that of the substrate. In the reverse direction, not only oxaloacetate, but also malonate, tartronate and fluorocitrate inhibited at concentrations less than that of the substrate. Fluorocitrate was the only compound tested which inhibited malate synthesis at concentrations less than those at which it inhibited malate decarboxylation. The three C₃-dicarboxylic acids (malonic acid, tartronic acid and mesoxalic acid) inhibited the decarboxylation reaction to approximately the same extent,

but they were inhibitory to malate synthesis at widely differing concentrations. Also, in this latter direction, it appeared that the mechanism of the inhibition was different since the malonate inhibition was partially competitive, the tartronate inhibition non-competitive and that by mesoxalate partially uncompetitive. No explanation can be offered for these findings.

The decarboxylation of malate is not very specific in cation requirements, Mn^{2+} , Mg^{2+} and Co^{2+} ions, all activating at the same concentration (Stickland, 1959). For malate synthesis Mn^{2+} ions were much more efficient than the others, giving a maximum rate at 1% of the concentration of Mg^{2+} or Co^{2+} ions required. The Mn^{2+} ion (unhydrated) has a radius of 0.80 Å; the radii of the bivalent Mg^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} ions are 0.65–0.74 Å (values of Pauling, as quoted in Hodgman, 1950). The radius of the Mn^{2+} ion is thus greater than that of all the other ions whose effects on malate synthesis were studied, and this ion might fit the enzyme-substrate complex better. The inhibition by Zn^{2+} and Cu^{2+} ions is presumably due to their blocking the active centre.

The non-enzymic removal of TPNH formed by reduction of TPN^+ with dithionite is brought about only by metals that are members of the first transition period of the periodic table. Thus these metals all have a variable valency. The disappearance of TPNH is probably due to its oxidation, the ions acting catalytically. It is not clear why this oxidation should be affected by bicarbonate.

The K_m for pyruvate ($2 \times 10^{-2} M$) is very high. The pyruvate concentration in rat liver is approximately 0.6 mM (as calculated from the data of Frohman, Orten & Smith, 1951). Assuming it is about the same in pigeon liver, the malic enzyme is far from saturated with pyruvate. However, since the K_m is so much higher than the pyruvate concentration, the rate of malate synthesis will be approximately proportional to the pyruvate concentration.

SUMMARY

1. The synthesis of malate from pyruvate and bicarbonate by the malic enzyme of pigeon liver has been studied.

2. Malate synthesis was inhibited by carbon dioxide when the carbon dioxide pressure was above about 0.45 atmosphere. The apparent Michaelis constant for pyruvate was very high.

3. Bivalent metal ions were essential for the reaction. The same rate was given by 0.033 mM- Mn^{2+} as by 3.3 mM- Mg^{2+} or Co^{2+} ions. Zn^{2+} and Cu^{2+} ions were inhibitory.

4. In the presence of 10 mM-pyruvate, 40–50% inhibition was caused by tartronate (0.1–0.01 mM), fluorocitrate (0.1 mM), malonate (mM), L-malate (mM), oxaloacetate (1–10 mM), D- and L-lactate, DL- α -hydroxybutyrate and mesoxalate (all 20 mM). mesoTartrate and citrate were weaker inhibitors. Propionate and DL- β -hydroxybutyrate were not inhibitory.

5. Triphosphopyridine nucleotide which had been reduced by dithionite was non-enzymically oxidized in the presence of Mn^{2+} , Co^{2+} , Ni^{2+} or Cu^{2+} ions. Enzymically reduced triphosphopyridine nucleotide did not show this phenomenon unless sulphite was added. The oxidation in the presence of Cu^{2+} ions was inhibited by bicarbonate; that by the other ions was increased by bicarbonate.

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