The Oxidation of Malic and *meso*Tartaric Acids in Pigeon-liver Extracts

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Malic acid is oxidized by malic dehydrogenase and by the malic enzyme, both enzymes requiring the presence of diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN). In the course of experiments on the malic enzyme of pigeon liver, a fraction has been obtained which oxidized sodium DL-malate in the presence of ferricyanide but for which no requirement for DPN, TPN or manganous ions could be shown. The kinetics of this oxidation and its inhibition by other hydroxy acids have been investigated.

Although an effect of *meso*tartaric acid on malic dehydrogenase has not been reported, it is known to affect succinic and lactic dehydrogenase activity in *Escherichia coli* (Quastel & Wooldridge, 1928) and to have some inhibitory action on prostate phosphatase (Anagnostopoulos, 1953). Tartronic (hydroxymalonic) acid, a homologue of malic acid, inhibits malic dehydrogenase (Green, 1936), a concentration of 0·06M producing 24% inhibition in pig-heart extracts at an unspecified substrate concentration. Tartronic acid also inhibits the lactic dehydrogenase activity of *Esch. coli* but, in contrast to *meso*tartaric acid, has no effect on the succinic dehydrogenase (Quastel & Wooldridge, 1928). It is also an effective inhibitor of lactate oxidation and anaerobic glycolysis but has no effect on pyruvate oxidation in guinea pig brain slices (Jowett & Quastel, 1937).

MATERIALS AND METHODS

Sodium hydrogen DL-*malate.* This was recrystallized 3 times from 80% (v/v) aqueous ethanol and before use was brought to pH 7·4 with NaOH.

*meso*Tartaric *acid.* A sample from British Drug Houses Ltd., was used without further purification.

DPN and TPN. These were commercial samples obtained from Schwarz Laboratories and Sigma Chemical Co., respectively. Assay of the DPN indicated a purity of 55%.

Pigeon-liver extract. This was prepared by the method of Mehler, Kornberg, Grisolia & Ochoa (1948). The crude preparation was used, consisting of a dialysed, $(NH_4)_2SO_4$-fractionated extract of an acetone powder.

Standard manometric procedures were adopted, with ferricyanide (Quastel & Wheatley, 1938) and an atmosphere of 93% N_2–7% CO_2 at 37°. Substrates and inhibitors were tipped into the Warburg flasks after 3 min. incubation and after a further 3 min. initial readings were recorded.

RESULTS

The oxidation of malate in pigeon-liver extracts

Preliminary experiments confirmed the presence in the pigeon-liver extract of a manganese-requiring TPN-linked malic enzyme dependent upon the presence of a hydrogen-acceptor system such as pyruvate-lactic dehydrogenase (Mehler *et al.* 1948). When attempts were made to substitute ferricyanide for the acceptor system it was found that anaerobic oxidation of malate occurred in the absence of added DPN, TPN or manganese (see Table 1). In the same system no oxidation of *iso*-citrate occurred except in the presence of added TPN and none of lactate or β-hydroxybutyrate except in the presence of added DPN. The oxidation of malate, therefore, did not proceed via a soluble pyridine nucleotide in the extract itself.

Since the oxidation of malate could not be due to the presence of malic dehydrogenase, it

Table 1. *The anaerobic oxidation of* DL-*malate and* meso*tartrate in pigeon-liver extract*

All flasks contained 0·5 ml. of 1·3% sodium bicarbonate, 0·2 ml. of 3% $K_3Fe(CN)_6$ (higher concentrations gave a precipitate in the presence of added Mn^{2+}), 1·0 ml. of pigeon-liver extract, 0·3 ml. of 0·2M sodium DL-malate or 0·2M sodium *meso*tartrate and water to make a final vol. of 3 ml.

Additions			CO_2 evolved in 20 min.	
Mn^{2+} (μatoms)	DPN (mg.)	TPN (mg.)	DL-Malate (μl.)	*meso*Tartrate (μl.)
0·9	0	0·8	123	59
0	0	0·8	127	55
0·9	0	0	127	13
0·9	1·0	0	126	85
0	1·0	0	128	85

was felt that a determination of the Michaelis constant might serve to characterize the enzyme system. The K_m value obtained for DL-malate was $6{\cdot}2 \times 10^{-3}$M or $3{\cdot}1 \times 10^{-3}$M for the L isomer (see Figs. 1 and 2). The value obtained for the pig-heart enzyme was 10^{-2}M and approximately the same value has been reported for both the L-malic dehydrogenase of washed suspensions of *Esch. coli* (Gale & Stephenson, 1939) and the malic enzyme of bacteria (Korkes, Del Campillo & Ochoa, 1950).

The effect of other hydroxy acids on the anaerobic oxidation of DL-*malate in pigeon-liver extracts*

It has been shown (Green, 1936) that 0·06M tartronic acid inhibits malate oxidation by the pig-heart enzyme by 24%. When the effect of tartronic acid on malate oxidation in the pigeon-liver extract was examined and the results analysed by the method of Lineweaver & Burk (1934), it was found that tartronate was a competitive inhibitor of DL-malate oxidation and had $K_I = 9 \times 10^{-5}$M (Fig. 1). From this value it is estimated that tartronic acid is approximately 1000 times as active an inhibitor in the pigeon-liver system as in the pig-heart system.

Furthermore, maleate does not inhibit malate oxidation in pig heart (Green, 1936), but at a concentration of 0·02M it inhibited the oxidation of 0·02M DL-malate in the pigeon-liver system by 50%. Similarly, *iso*citrate (55% at 0·01M) and malonate (30% at 0·02M) both inhibited the oxidation of 0·02M DL-malate under the present conditions. Butyrate, β-hydroxybutyrate and gluconate, however, had no effect on malate oxidation.

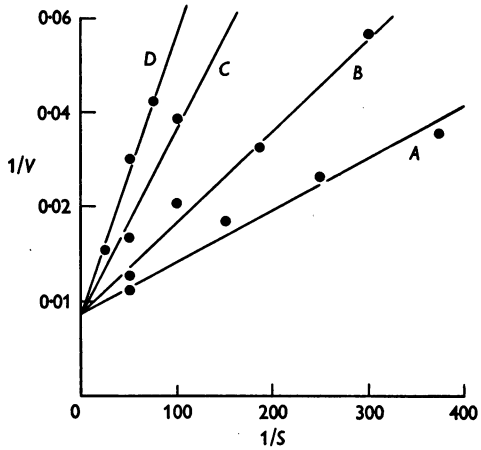

Fig. 1. The competitive inhibition by tartronate of the oxidation of DL-malate in pigeon-liver extracts. Conditions as in Table 1. *A*, no inhibitor; *B*, $6{\cdot}7 \times 10^{-5}$M Na tartronate; *C*, $2{\cdot}2 \times 10^{-4}$M Na tartronate; *D*, $6{\cdot}7 \times 10^{-4}$M Na tartronate; *S* = molar substrate concentration; *V* = velocity, expressed as μl. CO_2 evolved in 32 min.

Citrate was found to be an effective inhibitor, and the results, analysed by the method of Lineweaver & Burk (1934), showed that the inhibition was competitive (Fig. 2) with $K_I = 2{\cdot}65 \times 10^{-3}$M. Sodium D-tartrate and DL-tartrate did not inhibit the anaerobic oxidation of DL-malate, but sodium *meso*-tartrate proved to be a competitive inhibitor with $K_I = 3{\cdot}2 \times 10^{-3}$M. In this connexion, it is interesting to note that the stereoisomers of tartaric acid do not have the same action on prostate phosphatase. This enzyme is strongly inhibited by the D isomer, feebly inhibited by the *meso* isomer and stimulated by the L isomer (Anagnostopoulos, 1953).

The oxidation of meso*tartrate in pigeon-liver extracts*

The particular fraction of pigeon-liver extract under investigation does not oxidize tartronic, citric, *iso*citric, gluconic, maleic, succinic, malonic, butyric, D-tartaric or DL-tartaric acids in the presence or absence of DPN with ferricyanide as hydrogen acceptor. It does oxidize *iso*citric acid in the presence of TPN and shows some activity towards lactate and β-hydroxybutyrate in the presence of DPN, but the rate of oxidation in all cases falls off rapidly owing to accumulation of the products in the absence of fixatives.

However, in the presence of DPN or TPN, *meso*-tartaric acid was oxidized anaerobically by the pigeon liver–ferricyanide system (Table 1). Manganese ions had no effect on the rate of oxidation.

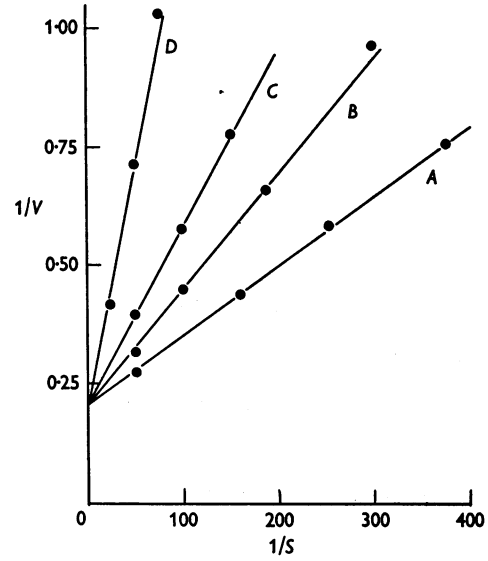

Fig. 2. The competitive inhibition by citrate of the oxidation of DL-malate in pigeon-liver extracts at pH 7·4. Conditions as in Table 1. *A*, no inhibitor; *B*, $1{\cdot}67 \times 10^{-3}$M Na citrate; *C*, 4×10^{-3}M Na citrate; *D*, $1{\cdot}33 \times 10^{-2}$M Na citrate; *S* = molar substrate concentration; *V* = initial velocity, expressed as μl. CO_2 evolved/min.

The K_m value for *meso*tartaric acid in this system is high, $1{\cdot}4 \times 10^{-2}$ M, compared with the K_m value for DL-malate, $6{\cdot}2 \times 10^{-3}$ M. The K_m value found for DPN was approximately 5×10^{-3} M. None of the carboxylic acids tested as potential inhibitors of DL-malate oxidation were found to inhibit *meso*tartaric acid oxidation in pigeon-liver extracts.

Similarly, addition of *meso*tartrate to rat-liver mitochondria resulted in an increased rate of oxygen uptake but much lower activities were found with rat-kidney mitochondria.

DISCUSSION

Sodium DL-malate is oxidized anaerobically by ferricyanide in the presence of pigeon-liver extracts. The oxidation is not catalysed by the malic enzyme since it could be shown that in these extracts TPN is essential for malic enzyme activity, whereas no coenzyme requirement for anaerobic oxidation by ferricyanide could be demonstrated. Further, the marked inhibition of this oxidation by tartronic and maleic acids and the much lower K_m value are not in agreement with the properties of the malic dehydrogenase of pig-heart extracts or resting suspensions of *Esch. coli*. It seems most likely that the responsible enzyme is similar to malic dehydrogenase but with physical constants differing from those found for the enzyme obtained from other sources. A binding of coenzyme by the dehydrogenase similar to that of DPN by glyceraldehyde 3-phosphate dehydrogenase (Velick, Hayes & Harting, 1953) may occur, however, and thus obviate the need for additional coenzyme.

This dehydrogenase is inhibited by *meso*tartaric, tartronic, maleic, malonic, *iso*citric and citric acids, and the inhibition by *meso*tartaric, tartronic and citric acids has been shown to be competitive. The K_I values found are not in agreement with those reported for L-malic dehydrogenase or the malic enzyme of various origins.

*meso*Tartaric acid is oxidized in pigeon-liver extract by a dehydrogenase which requires the presence of either DPN or TPN. With DL-malate or *meso*tartrate oxidation, the products do not seem to inhibit the reaction to a great extent since, in general, the initial velocities are maintained. In contrast, little oxidation of lactate or *iso*citrate could be obtained in the absence of a fixative. The dehydrogenase oxidizing *meso*tartaric acid is not the same as that oxidizing malate since it has a demonstrable DPN or TPN requirement and is not inhibited by any of the compounds shown to inhibit malate oxidation in the same system.

SUMMARY

The kinetics of a characteristic malic dehydrogenase of pigeon-liver extract have been investigated and a DPN-linked dehydrogenase oxidizing *meso*-tartrate in the same system is described.

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Antigenicity of Insulin: Diabetes Induced by Specific Antibodies

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Lewis (1937) sensitized guinea pigs with ox or pig insulin. Using the Dale technique, he observed contractions of uterine strips from these animals in response to both ox and pig insulin. There was a more persistent response to the preparation of insulin which had been used to sensitize the animal. Bernstein, Kirsner & Turner (1938) have reported anaphylaxis in guinea pigs sensitized and challenged with various insulin preparations. Wasserman & Mirsky (1942) showed that anaphylaxis could be induced in guinea pigs with insulin of a different species than that used for sensitization. Banting, Franks & Gairns (1938) reported the development of resistance to insulin in a non-diabetic schizophrenic patient following a course of insulin shock treatment. Blood serum from this patient, mixed with