The Enzymic Conversion of the Tartaric Acids to Oxaloacetic Acid

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SUMMARY: Certain bacteria of the genus *Pseudomonas* attack the tartaric acids by means of inducible stereospecific dehydrases. Each dehydrase converts its specific isomeric substrate to oxaloacetic acid; in crude cell-free extracts the oxaloacetic acid is in turn converted to pyruvic acid, which accumulates. By treatment of the crude extracts with ethylenediaminetetraacetic acid (EDTA), substantial accumulations of oxaloacetic acid can be obtained from the *meso*- and *d*-tartaric acids, and assays for these two dehydrases, based on the accumulation of oxaloacetic acid in the presence of EDTA, have been developed. This procedure cannot be used to assay the *l*-tartaric acid dehydrase, which is itself very sensitive to EDTA. The patterns of inhibition of the three dehydrases by compounds sterically related to their substrates have been explored, and the findings are interpreted in terms of the minimal steric requirements for enzyme-substrate combination.

Many micro-organisms can attack the enantiomorphs of tartaric acid, usually with some degree of steric specificity (Pasteur, 1860, 1863; Barker, 1936; Vaughn, Marsh, Stadtman & Cantino, 1946; Stadtman, Vaughn & Marsh, 1945; Tabachnick & Vaughn, 1948; Mercer & Vaughn, 1951; Nomura & Sakaguchi, 1955). Surprisingly, there is only one brief report of microbial attack on *meso*-tartaric acid (Martin & Foster, 1955). Until recently, the biochemical mechanisms involved in the metabolism of the tartaric acids have been unknown. Barker (1936) proposed that the dissimilation of *d*-tartaric acid by *Aerobacter aerogenes* proceeds through oxaloacetate and pyruvate. On the basis of somewhat indirect evidence, the occurrence of this pathway in fluorescent pseudomonads was suggested by Nomura & Sakaguchi (1955) and by la Rivière (1956). While the work reported here was in progress, it was shown by Krampitz & Lynen (1956) that the dissimilation of *d*-tartaric acid by an unidentified Gram-positive rod involves a primary dehydration to oxaloacetic acid. A different pathway for the metabolism of the *l*- and *meso*-tartaric acids exists in animal mitochondria (Scholefield, 1955; Kun & Hernandez, 1956; Kun & Davies, 1956; Kun, 1956). Here, the primary attack occurs by dehydrogenation to oxaloglycollic acid.

This paper describes the enzymic mechanisms of tartaric acid metabolism by a series of *Pseudomonas* strains, which differ in the specificity of their attack on the three isomers. The existence of strains completely specific for a single isomer greatly facilitated the investigation of this problem. In an accompanying paper (Shilo & Stanier, 1957), certain physiological properties of the organisms concerned are described.

Configurational notation

In the chemical literature, the configurations assigned to the enantiomorphs of tartaric acid are inconsistent: some authors designate the (+)-isomer as L-(+)-tartaric acid, others as D-(+)-tartaric acid. As Buchanan (1951) pointed out, this ambiguity is an inevitable consequence of the current configurational notation. From the reference standard, D-(+)-glyceraldehyde, one can derive (-)-tartaric acid, via D-(-)-threose; but one can also derive (+)-tartaric acid, via D-(-)-glyceric acid, (+)-isoserine and (+)-malic acid. This confused situation can only be remedied by the adoption of a new series of conventions for the specification of configuration, such as those proposed by Cahn & Ingold (1951). In the meantime, the biologist is faced with a dilemma. We shall resolve it by referring to the tartaric acids and the related substances discussed in this and the ensuing paper in terms of their rotations, as *d*-tartaric acid, *d*-malic acid, etc. The configurational relationships, using the Fischer convention, are indicated by the following structures:

METHODS

Organisms and conditions of cultivation. *Pseudomonas* strains *meso* 1, *d* 15 and *l* 1 were used. They were isolated by elective culture with *meso*-, *d*- and *l*-tartaric acids, respectively. In all cases, the attack on the isomers of tartaric acid was strictly and specifically inducible. Strains *meso* 1 and *d* 15 can attack only *meso*- and *d*-tartaric acid, respectively. Strain *l* 1 can be induced to attack either *d*- or *l*-tartaric acid, by growth in the presence of the appropriate isomer.

The basal medium used for the preparation of specifically induced organisms consisted of: Na tartrate, 5.0 g.; NH_4Cl , 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g.; FeCl_3 , 0.05 g.; Difco yeast extract, 0.025 g.; phosphate buffer (0.01 M, pH 7.2), 1000 ml. Cultures were incubated at 30° on a mechanical agitator in Fernbach flasks (2 l. capacity) containing 1000 ml. medium. Organisms were harvested before termination of the exponential phase of growth.

Chemicals. *d*-Malic acid (the unnatural isomer) and *d*-, *l*- and *meso*-isomers of tartaric acid were purchased from the California Foundation for Biochemical Research. The *cis* and *trans* isomers of *dl*-epoxysuccinic acid were kindly furnished by Dr J. W. Foster.

Preparation of extracts. The organisms from 1 l. of culture medium were harvested by centrifugation, washed once with phosphate buffer (pH 7.2,

0.02 M), and resuspended at a density of 4–5 % (w/v) in the same buffer. This suspension was then disintegrated by treatment for 10 min. in an ice-cooled Raytheon 9-KC sonic oscillator. Coarse debris and the particulate fraction were sedimented by centrifugation for 30 min. at 19,000 g, yielding a clear straw-coloured supernatant liquid which contained c. 10 mg. protein/ml. Such extracts were used in all the experiments to be described.

Chemical methods. Pyruvic acid and total keto acids were estimated colorimetrically by the method of Friedman & Haugen (1943), and chromatographically by the method of Cavallini, Frontali & Toshi (1949). β -Ketoacids were measured manometrically by catalytic decarboxylation with 4-aminoantipyrine (Akamatsu, 1950; Sistrom & Stanier, 1953). Oxaloacetic was determined quantitatively by light absorption at 280 m μ . with a Beckman model DU spectrophotometer (Green, Leloir & Nocito, 1945; Cammarata & Cohen, 1951). *d*- and *l*-Tartaric acids were estimated colorimetrically by the metavanadate method (Matchett, Legault, Nimmo & Notter, 1944), and detected chromatographically on Whatman no. 1 paper after development with butanol/water/acetic acid mixture (50:40:10) by spraying with bromphenol blue. Protein estimations were made by Pardee's modification of the biuret method (Pardee, personal communication). Manometric measurements of oxygen uptake and CO₂ evolution were carried out in the conventional Warburg apparatus at 30° with air as the gas phase.

RESULTS

The formation of oxaloacetic acid and pyruvic acid from the tartaric acids

Crude extracts, centrifuged at gravitational fields insufficient to sediment all the cytochrome-bearing particles, can oxidize specifically the tartaric acid isomers for which the bacteria were induced; however, the very high endogenous respiration makes the data difficult to interpret. When extracts were thoroughly clarified by centrifugation, they showed a very low endogenous oxygen consumption, which was only slightly increased by the addition of tartaric acid. However, with such extracts a rapid evolution of CO₂ resulted from the addition of the appropriate isomer of tartaric acid; the total amount of CO₂ evolved amounted to slightly more than one mole/mole tartaric acid supplied.

Typical manometric data, obtained with an extract from strain *meso* 1 acting upon *meso*-tartaric acid, are shown in Fig. 1. The same type of manometric data was obtained with extracts of strains *d* 15 and *l* 1, acting upon the *d*- and *l*-isomers, respectively. Chromatographic analysis showed that the tartaric acid furnished had disappeared completely from the reaction mixture at the time when the evolution of CO₂ had ceased. These observations establish that the primary attack on all three isomers is a non-oxidative one. In each case, chemical analysis of the reaction mixture showed that pyruvic acid had accumulated in appreciable quantities; it was the only major keto-acid present (Table 1).

The formation of CO₂ and pyruvic acid from tartaric acid suggested that the

first product formed might be oxaloacetic acid, which did not accumulate because of its rapid decarboxylation. Accordingly, the effect of ethylenediamine tetraacetic acid (EDTA) on tartrate breakdown was tested. Since EDTA chelates Mg^{++} , it might be expected to inhibit the decarboxylation of oxaloacetic acid.

Table 1. *The formation of pyruvic acid from the three isomers of tartaric acid in the presence of specific bacterial extracts*

The incubation mixtures contained 100 μ mole sodium tartrate and 0.5 ml. of enzyme extract in phosphate buffer (0.02 M, pH 7.2) in a total volume of 2 ml. The mixtures were incubated at 30° for 10 min., after which the reactions were stopped by adding 2 ml. of 10 % trichloroacetic acid. Pyruvate was estimated in the deproteinized supernatant fluid by the Friedman & Haugen (1943) method, with xylene for extraction of the ketoacid.

Substrate	Source of extract (strain)	Pyruvate found (μ mole)	E420/E520
<i>d</i> -Tartaric acid	<i>d</i> 15	12.7	1.37
<i>l</i> -Tartaric acid	<i>l</i> 1	11.6	1.38
<i>meso</i> -Tartaric acid	<i>meso</i> 1	26.7	1.31

The addition of EDTA (final concentration 0.04 M) to an extract from strain *meso* 1 virtually abolished the evolution of CO_2 from *meso*-tartaric acid (Fig. 2). This was accompanied by the accumulation of large quantities of β -ketoacid, as shown by the large amount of CO_2 evolved upon addition of 4-aminoantipyrine to the reaction mixture after 15 min. of incubation; in the absence of EDTA, the accumulation of β -ketoacid was negligible (Fig. 2). The 2,4-dinitrophenyl hydrazones of the ketoacids accumulating from *meso*-tartrate in the presence and absence of EDTA were prepared and subjected to chromatographic analysis. The reaction mixture without EDTA yielded only the 2,4-dinitrophenylhydrazone of pyruvic acid; the reaction mixture with EDTA yielded also a second 2,4-dinitrophenylhydrazone, chromatographically indistinguishable from the 2,4-dinitrophenylhydrazone of oxaloacetic acid. Furthermore, analysis by the method of Friedemann & Haugen showed that in the extract treated with EDTA, the total quantity of ketoacid was almost double the quantity of pyruvic acid (Table 2).

Table 2. *The accumulation of ketoacids from meso-tartaric acid in the presence and absence of EDTA*

The incubation mixtures contained: 20 μ mole *meso*-tartaric acid; 0.5 ml. of *meso* 1 extract; and phosphate buffer (0.02 M, pH 7.2) to make 1.5 ml. The reactions were stopped by the addition of 1.5 ml. of 10 % trichloroacetic acid after 15 min. of incubation at 30°. Pyruvate was estimated in the deproteinized supernatant by the method of Friedman & Haugen (1943) in xylene extracts. For total ketoacid estimation, ethyl acetate was used as solvent.

Treatment	Pyruvate (μ mole)	Total ketoacids (μ mole)
No EDTA added	18.0	17.4
10 μ mole EDTA	6.9	11.05

By similar methods, the extract from strain *d* 15 was shown to produce oxaloacetic acid from *d*-tartaric acid in the presence of 0.04 M EDTA. The extract from strain *l* 1, however, did not decompose tartaric acid in the presence of 0.04 M-EDTA. With lower concentrations of EDTA (0.004 M), the reaction proceeded and a small accumulation of oxaloacetic acid was demonstrated.

Fig. 1

Fig. 2

Fig. 1. O_2 uptake and CO_2 evolution from *meso*-tartaric acid by an extract of strain *meso* 1. Incubation mixture contained: 5 μ mole neutralized *meso*-tartrate; 0.5 ml. *meso* 1 extract; phosphate buffer (0.02 M, pH 7.2) to make 2 ml. Temperature 30°.

Fig. 2. Effect of EDTA on CO_2 evolution and β -ketoacid accumulation from *meso*-tartaric acid by an extract from strain *meso* 1. Incubation mixtures contained: 5 μ mole *meso*-tartaric acid; 0.5 ml. *meso* 1 extract; phosphate buffer (0.02 M, pH 7.2) to make 1.8 ml. Temperature 30°. At points indicated by arrows, the incubation mixture was acidified with 0.1 ml. *N*-acetic acid and 0.4 ml. 4-aminoantipyrine (0.1 M) was added.

It is accordingly evident that the decomposition of the three isomers of tartaric acid involves in each case a specific dehydration to oxaloacetic acid (probably the enol form), followed by decarboxylation of the oxaloacetic acid to pyruvic acid. Thus only the initial step in the metabolism of the tartaric acids is biochemically specific and unique; with the formation of oxaloacetic acid and pyruvic acid, the main pathways of intermediary metabolism are attained. We shall term the specific enzymes involved tartaric acid dehydrases.

The development of assays for the tartaric acid dehydrases

Oxaloacetic acid can be estimated spectrophotometrically by its light absorption at 280 $m\mu$. Since it accumulates when *meso*-tartaric acid is enzymically attacked in the presence of EDTA, a spectrophotometric assay which measures the accumulation of oxaloacetic acid directly was developed. Fig. 3 shows the accumulation of oxaloacetic acid in the presence and in the absence of EDTA (10 μ mole) as estimated from optical density changes at 280 $m\mu$.

In the absence of EDTA, there was an initial rapid formation of oxaloacetic acid from *meso*-tartaric acid, followed by a decline. The maximal quantity of oxaloacetic acid accumulated from 5 μ mole *meso*-tartrate was less than 2 μ mole. In the presence of EDTA, oxaloacetic acid accumulated steadily, more than 4 μ mole being formed from 5 μ mole *meso*-tartrate, and more than 8 μ mole from 10 μ mole *meso*-tartrate. At the points indicated by arrows in

Fig. 3

Fig. 4

Fig. 3. Effect of EDTA on the accumulation of oxaloacetic acid from *meso*-tartaric acid by an extract from strain *meso* 1. An incubation mixture contained 0.05 ml. *meso* 1 extract and phosphate buffer (0.02 M, pH 7.2) to make 2.5 ml., ▲—▲, 5 μ mole *meso*-tartrate; ●—●, 5 μ mole *meso*-tartrate + 10 μ mole EDTA; Δ—Δ, 10 μ mole *meso*-tartrate + 10 μ mole EDTA. The accumulation of oxaloacetic acid was followed at 280 m μ . in the Beckman spectrophotometer. Mg⁺⁺ (10 μ mole) was added at points indicated by arrows.

Fig. 4. The effect of enzyme concentration on the rate of accumulation of oxaloacetic acid from *meso*-tartaric acid. Incubation mixtures containing 10 μ mole of neutralized *meso*-tartaric acid, 10 μ mole EDTA, varying amounts of *meso* 1 extract and phosphate buffer (0.02 M, pH 7.2) were tested for differences in the rate of accumulation of oxaloacetic acid, as measured in the Beckman spectrophotometer at 280 m μ .

Fig. 3, 10 μ mole of MgCl₂ were added to the reaction mixtures. In each case there was an immediate rapid rise in optical density, a phenomenon which is also observed when Mg⁺⁺ is added to neutral aqueous solutions of pure oxaloacetic acid. The explanation for this is unknown, but it possibly reflects formation of a magnesium-complex with oxaloacetic acid. Following this initial increase in optical density, the optical density of the reaction mixture fell rapidly to a negligible value, reflecting the decarboxylation of oxaloacetic acid to pyruvic acid.

From Fig. 3, it can be seen that with a given quantity of enzyme, the initial rate of accumulation of oxaloacetic acid in the presence of EDTA was virtually identical for 5 and 10 μ mole *meso*-tartrate. The effect of enzyme concentration on the initial rate of formation of oxaloacetic acid in the presence of EDTA is shown in Fig. 4; within limits, there is a linear relationship between enzyme concentration and activity. Hence it is evident that the spectrophotometric method provides a convenient continuous assay procedure for the *meso*-tartaric acid dehydrase in the presence of EDTA. With *d*-tartaric acid dehydrase, the same assay method can be used, although the accumulation of oxaloacetic acid is never so complete, and the rate of its formation starts to

Fig. 5. Effect of EDTA on *l*-tartrate dehydrase. Incubation mixtures contained: 0.04 ml. extract of strain *l* 1 grown on *l*-tartrate medium; 10 μ mole *l*-tartrate; different amounts (0 to 3 μ mole) of EDTA; phosphate buffer (0.02 M, pH 7.2) to make 2.5 ml. At times indicated by arrows, 10 μ mole of Mg^{++} were added. Accumulation of oxaloacetic acid was followed in the Beckman spectrophotometer at 280 $m\mu$.

decline at an earlier point in the reaction. Although the accumulation of oxaloacetic acid from *l*-tartaric acid in the presence of the specific dehydrase can be demonstrated, this dehydrase is itself strongly inhibited by EDTA (Fig. 5). The assay of this enzyme can be made, although somewhat imprecisely, by measuring the initial rate of accumulation of oxaloacetic acid in a reaction system without EDTA.

General properties of the tartaric acid dehydrases

The stereospecificity of the three tartaric acid dehydrases is absolute: each enzyme is capable of attacking only one isomer of tartaric acid. Furthermore, no other substrates for any of these enzymes have been found. The reactions catalysed by the *meso*- and *d*-tartaric acid dehydrases appear to be essentially irreversible: when the enzymes preparations were incubated with oxaloacetic acid, the decrease in optical density at 280 $m\mu$. was not greater than that observed in controls containing oxaloacetic acid alone. The reversibility of the reaction catalysed by the *l*-tartaric acid dehydrase cannot be tested in this way, since the decarboxylation of oxaloacetic acid cannot be blocked by EDTA treatment.

Experiments with the dehydrases were usually conducted at pH 7.2 in phosphate buffer. The effect of pH value on the activity of the *meso*-tartaric acid dehydrase was measured; there was a broad plateau between pH 6.9 and 8.5, with a rapid decline of activity on both sides.

Attempts to purify the *meso* dehydrase have so far failed. Dialysis and ammonium sulphate precipitation both caused great loss of activity, somewhat decreased by the presence of EDTA. Nevertheless, in crude extracts the *meso* dehydrase is stable for several weeks when kept frozen. The *d*- and *l*-tartaric acid dehydrases, on the other hand, lost considerable activity when stored for a few days in the frozen state.

Table 3. *Patterns of inhibition of the tartaric acid dehydrases by sterically related compounds*

The incubation mixtures contained: 10 μ mole of specific substrate, 10 μ mole of the listed compound, and phosphate buffer (0.02 M, pH 7.2) to make 2.5 ml. In the cases of the *meso* dehydrase and the *d* dehydrase, 10 μ mole of EDTA were also added. The rate of accumulation of oxaloacetic acid was estimated at 280 m μ by the Beckman spectrophotometer, in the presence and absence of the compounds tested as inhibitors.

Inhibitor	% inhibition at an equimolar ratio to substrate		
	<i>Meso</i> dehydrase	<i>d</i> dehydrase	<i>l</i> dehydrase
<i>meso</i> -Tartaric acid	—	54	93
<i>d</i> -Tartaric acid	74	—	35
<i>l</i> -Tartaric acid	0	0	—
Citraconic acid	0	—	—
<i>d</i> , <i>l</i> -Citromalic acid	0	—	—
Itaconic acid	0	—	—
<i>d</i> , <i>l</i> -trans-Epoxy succinic acid	16	56	88
<i>d</i> , <i>l</i> -cis-Epoxy succinic acid	87	0	16
Tartronic acid	42	66	—
<i>d</i> -Malic acid	74	72	—
<i>l</i> -Malic acid	0	0	—

Inhibition pattern. In addition to their stereospecificity with respect to substrate, the three tartaric acid dehydrases showed characteristic individual patterns of inhibition by compounds sterically related to the substrates (see Table 3). The data were obtained by determining the decrease in the rate of oxaloacetic acid accumulation which occurred when each of the listed inhibitors was added to the enzyme preparation in the presence of an equimolar quantity of the specific substrate. It can be seen that in some cases the degree of inhibition was exceedingly severe: for example, the *meso* dehydrase was inhibited to the extent of at least 74 % by *d*-tartaric, *d*-malic and *d*, *l*-cis-epoxy-succinic acids, and the *l*-tartaric acid dehydrase was inhibited to an extent of 85 % or more by *meso*-tartaric acid and *d*, *l*-trans-epoxy-succinic acid. It seems likely that these inhibitors act competitively, although this has been definitely shown only for the inhibition of the *meso*-tartaric acid dehydrase by *d*-tartaric and tartronic acids.

DISCUSSION

The enzymic dehydration of tartaric acid to oxaloacetic acid, first established by Krampitz & Lynen (1956) for the *d*-isomer, occurs also with the *meso*- and *l*-isomers, and the attack on all three tartaric acids by bacteria of the genus *Pseudomonas* appears to occur principally by means of stereospecific dehydrases. In the present enzymic study, the existence of such stereospecific dehydrases was established for three strains of *Pseudomonas*. In addition, however, we have examined extracts from many other *Pseudomonas* strains which can attack one, two, or all three of the tartaric acids; in most cases oxaloacetic acid is an intermediate. This preliminary survey has shown, however, that at least one additional pathway for the metabolism of *d*-tartaric acid exists in pseudomonads. Extracts from certain *d*-specific strains did not yield oxaloacetic acid from *d*-tartaric acid in the presence of EDTA; instead, small amounts of a ketoacid similar in its properties to glyoxylic acid accumulated.

The patterns of inhibition observed with the three tartaric acid dehydrases can be interpreted, at least in part, in steric terms. We shall consider first the inhibitory interactions between the three tartaric acids. *meso*-Tartaric acid, the internally compensated molecular form, can be regarded as containing both the *d* and the *l* configurations. The fact that the *meso* dehydrase is strongly inhibited by the *d*-isomer of tartaric acid, but not at all by the *l*-isomer, suggests that it is that portion of the *meso*-tartaric acid molecule that contains the *d*-configuration which is actually bound to the *meso* dehydrase. The *d* dehydrase is inhibited by the *meso*-isomer but not by the *l*-isomer, while the *l* dehydrase is inhibited by the *meso*-isomer but not by the *d*-isomer. These facts suggest that the combining sites of the tartrate dehydrases are specific for the enantiomorphic configuration shown by their respective substrates; and, further, that only one of the asymmetric carbons of the substrate is involved in the enzyme-substrate combination.

These interpretations of the minimal steric configurations necessary for binding by the three dehydrases also fit the observed inhibitions with other substances sterically related to the tartaric acids. Thus *d*-malic acid inhibits the *meso* dehydrase and the *d* dehydrase. Tartronic acid, which has the essential structure of the postulated combining region of the substrates, but lacks asymmetry, also inhibits the *meso* and *d* dehydrases. Lastly, there are the *trans*- and *cis*-epoxysuccinic acids to be considered. The *dl*-*cis* form strongly inhibits only the *meso* dehydrase, while the *dl*-*trans* form strongly inhibits the *d* and *l* dehydrases, but only slightly the *meso* dehydrase. Here it seems evident that geometrical configurations also play a role in the inhibitory process; until the enantiomorphs of each geometrical isomer have been tested separately, a detailed analysis in steric terms cannot be made.

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