Some Properties of Mandelate Racemase from *Pseudomonas fluorescens*

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1. L-Mandelate dehydrogenase and mandelate racemase were partially purified from extracts of *Pseudomonas fluorescens* A–312 grown in media containing D-mandelate. 2. The activity of mandelate racemase, but not that of L-mandelate dehydrogenase, is greatly stimulated by Mg^{2+}, Mn^{2+}, Co^{2+} and, though less effectively, by Ni^{2+}. Other metal ions are inactive or inhibitory. 3. Racemase activity is inhibited by phosphate, fluoride, pyrophosphate and EDTA. The inhibitions by pyrophosphate and EDTA are competitive with respect to the metal ion activator; those by phosphate and fluoride are competitive with respect to the substrate. 4. The addition of Mg^{2+} diminishes the Michaelis constant of racemase. 5. The pH optimum of the racemase is at 7·8. The pH–activity curve of the dehydrogenase complex of enzymes has two peaks, at 7·0 and 8·2. 6. The enzymic racemization of D-mandelate is initially faster than that of L-mandelate. 7. The rates of oxidation of related substrates, catalysed by L-mandelate dehydrogenase, are in the decreasing order: L-*p*-hydroxymandelate; L-3,4-dihydroxymandelate; L-4-hydroxy-3-methoxymandelate. The racemase is active towards D-*p*-hydroxymandelate but inactive towards D-3,4-dihydroxymandelate and D-4-hydroxy-3-methoxymandelate. Since 4-hydroxy-3-methoxymandelate, and presumably also 3,4-dihydroxymandelate, arising from the metabolism of catecholamines, have the D-configuration, the enzymes studied cannot be utilized for estimation of the last two acids in urine.

The classical work of Stanier and his associates (Stanier, 1950, 1955) has demonstrated the formation of a series of adaptive enzymes in *Pseudomonas fluorescens* grown in media containing mandelic acids. In the reaction sequence in which D-mandelic acid is broken down to catechol and beyond, the first two steps are catalysed by the enzymes mandelate racemase (EC 5.1.2.2) and L-mandelate dehydrogenase. As pointed out by Stanier (1955), little is known about these enzymes. They attracted my attention because of a publication by Rosano (1964), who claimed to have developed a method for the estimation of urinary 4-hydroxy-3-methoxymandelic acid (vanillyl mandelic acid) based on its oxidation by mandelate dehydrogenase. The present paper is mainly concerned with the substrate specificity and the metal ion requirements of mandelate racemase.

EXPERIMENTAL

Preparation of enzymes. Cells of *Ps. fluorescens* A–312 were maintained on agar plates and grown at 25–30° in a medium containing 1·5g. of KH_2PO_4, 2·5g. of $(NH_4)_2HPO_4$, 5g. of NaCl, 0·2g. of $MgSO_4,7H_2O$, 2·5mg. of $FeNH_4(SO_4)_2$ and 1·5g. of D-mandelic acid/l. and adjusted to pH7·0 with 5N-NH_3. An inoculum was prepared by transferring cells to eight 50ml. conical flasks each containing 20ml. of medium. After 48hr. incubation in a metabolic shaker the inoculum was added to 6l. of medium contained in two 5l. conical flasks. The mixture was stirred at high speed and the cells were centrifuged after 48hr. or, in a few cases, after 60hr., when the enzyme yield was highest; yields were about the same after 48hr. or 60hr. incubation. The centrifuged cells were washed three times with 1% (w/v) KCl, suspended in 20ml. of 0·1M-imidazole–HCl buffer, pH7·0, and passed twice through a French press (American Instrument Co., Silver Spring, Md., U.S.A.). Coarse particles were removed by centrifuging at 7000*g* for 15min. at 0°. The supernatant was then centrifuged in the no. 40 rotor of the Spinco model L ultracentrifuge at 100000*g* for 30min. After decanting, the supernatant was centrifuged a second time under the same conditions; this usually resulted in the formation of a clearly visible red deposit. The tubes were allowed to drain completely and were wiped on the inside with absorbent paper. The solid residues were suspended in a few millilitres of 0·1M-imidazole–HCl buffer, pH7·0, in 25% (v/v) glycerol and redispersed by expulsion through a 25-gauge hypodermic needle. The resulting suspension (the 'particulate fraction'),

diluted to 15ml. with imidazole–glycerol buffer and stored at −15°, served as the source of L-mandelate dehydrogenase and the associated electron-transport system. It usually contained about 60mg. of protein.

For the removal of nucleic acids from the soluble fraction protamine sulphate was used in a few early preparations by the method of Stanier (1955). This was later replaced by streptomycin sulphate (Oxenburgh & Snoswell, 1965) as follows: the supernatant solution was mixed with solid $(NH_4)_2SO_4$ to give 90% saturation, the protein precipitate was centrifuged, dissolved in 10ml. of 0·1M-imidazole–HCl buffer, pH7·0, and dialysed at 3° against 8l. of running 1·25mM-imidazole–HCl buffer, pH7·0, for about 17hr. The dialysed solution was then treated at pH7·0 with 10% streptomycin sulphate in the ratio 1ml./100mg. of protein. After centrifuging the E_{280}/E_{260} ratio was greater than 1·0 without appreciable loss of protein. The supernatant solution was brought to 44% saturation by the addition of 0·8vol. of saturated $(NH_4)_2SO_4$ solution, previously adjusted to pH7·0. Precipitation of protein started after $(NH_4)_2SO_4$ saturation had reached 33%. The precipitate was collected and dissolved in 10ml. of 0·1M-imidazole–HCl buffer, pH7·0, in 25% glycerol. This solution, which usually contained about 50mg. of protein, was stored at −15° and served as the source of mandelate racemase. One mg. of protein contained 0·8–0·9 standard unit of racemase (measured at room temperature) (International Union of Biochemistry, 1965).

Measurement of enzyme activities. L-Mandelate was converted into benzaldehyde by the particulate fraction in a reaction involving oxidation and decarboxylation. D-Mandelate was not attacked by the particulate fraction, or only very slowly, but addition of the soluble fraction resulted in its rapid oxidation. Racemase activity was therefore measured by the oxidation of D-mandelate in the presence of excess of particulate fraction and limiting amounts of soluble fraction. Benzaldehyde is a stable end product as long as nicotinamide nucleotides are absent (Stanier, 1955).

Enzyme activities were followed at room temperature (21±1°) by the increase in E_{250} with the aid of a Cary model 14 recording spectrophotometer. The reaction was started by the addition of the substrate and readings were taken at 3min. intervals for up to 24min. The rate of the uninhibited reaction remained approximately linear during this time. The molecular extinction coefficient of benzaldehyde at 250mμ, the wavelength of maximum absorption at pH7·0 and above, is 12300, and that of mandelate is 180; hence the conversion of 0·1μmole of mandelate/ml. into benzaldehyde is equivalent to a change in E_{250} of 1·212.

The oxidation of phenolic derivatives of mandelic acid was followed at the appropriate wavelength for the absorption maximum, at pH7·0, of the aldehyde formed, namely 330mμ for *p*-hydroxymandelate and 345mμ for both 4-hydroxy-3-methoxymandelate and 3,4-dihydroxymandelate.

In a few experiments the change in rotation on incubation of the soluble fraction alone with either D- or L-mandelate was directly observed with the aid of a Rudolph model 200 photoelectric polarimeter in a cell with a 1dm. light-path, at the 436mμ mercury line. Readings with this instrument were reproducible to ±0·005°.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) against a standard of bovine serum albumin.

The substrates used were purchased from Calbiochem, Los Angeles, Calif., U.S.A.

RESULTS

Effect of metal ions on enzyme activity. The conversion of D-mandelate into benzaldehyde by the complete enzyme system is greatly accelerated by the addition of Mg^{2+}. Mg^{2+} can be replaced by Mn^{2+}, Co^{2+} or, less effectively, Ni^{2+}, but other metal salts were inactive or inhibitory (Table 1). Certain metal ions produced precipitates (Hg^{2+}, Fe^{2+}, Pb^{2+}) or highly absorbing complexes (Cu^{2+}) and could therefore not be tested in my assay system. Replacement of Na^+ by tris or K^+ did not affect enzyme activity.

Co^{2+}, Mn^{2+} and Mg^{2+} showed half-maximal activity at concentrations of 18, 40 and 57μM respectively, and maximum activity at concentrations of 0·1, 0·5 and 3mM respectively (Fig. 1).

The oxidation of L-mandelate by the particulate fraction was not significantly stimulated by the addition of Mg^{2+}. It may be concluded therefore that the activating effect is on the racemase.

In the absence of Mg^{2+} the oxidation of D-mandelate shows an initial lag period that is largely eliminated by the addition of Mg^{2+}. Hence the stimulating effect of Mg^{2+} is highest at the begin-

Table 1. *Effects of multivalent cations on enzyme activity*

Each sample contained imidazole–HCl buffer, pH7·0 (83mM), D-mandelate (1mM), particulate fraction (167μg. of protein) and soluble fraction (90μg. of protein) in 3ml. Incubation was for 12min. at 21°.

Ion added	Concn. (mM)	Relative enzyme activity (control activity=100)
Mg^{2+}	3·0	208±6·65*
Mn^{2+}	0·5	232
	2·0	241
Co^{2+}	0·5	211
	2·0	186
Ni^{2+}	0·5	149
	2·0	122
Ca^{2+}	0·5	63
	2·0	51
Ba^{2+}	0·5	108
	2·0	51
Cd^{2+}	0·5	37·5
	2·0	30·5
Zn^{2+}	0·5	47·5
	2·0	5·3
Al^{3+}	0·5	101

* Mean ±S.E.M. of five experiments.

ning of the reaction and then gradually decreases (Fig. 2).

Effect of anions on enzyme activity. Phosphate, pyrophosphate, fluoride and EDTA inhibited the activity of the enzyme system. The concentrations required for 50% inhibition were approx. 0·1M, 1·5mM, 1·5mM and 0·10mM respectively. As was to be expected, the inhibitory effect of EDTA was reversed by addition of Mg^{2+} and it was thought likely that the effects of the other three anions would also prove to be due to chelation of Mg^{2+}. This, however, proved to be the case only for pyrophosphate. The inhibitions caused by fluoride and phosphate were independent of increasing concentrations of Mg^{2+} (Table 2). On the other hand, whereas the inhibitory effects of EDTA and pyrophosphate were not alleviated by increasing concentrations of D-mandelate, those of fluoride and phosphate were relieved thereby (Table 3); Lineweaver & Burk (1934) plots confirmed the competitive nature of the inhibition. It was necessary to pass the solutions of D-mandelate through a column of Dowex A–1 chelating resin (Na^+ form, 50–100 mesh) before using them in these experiments since unpurified solutions of D-mandelate, in concentrations above 1mM, counteracted the inhibitions by EDTA or pyrophosphate, presumably owing to the presence of activating metal ions.

The oxidation of L-mandelate by the particulate

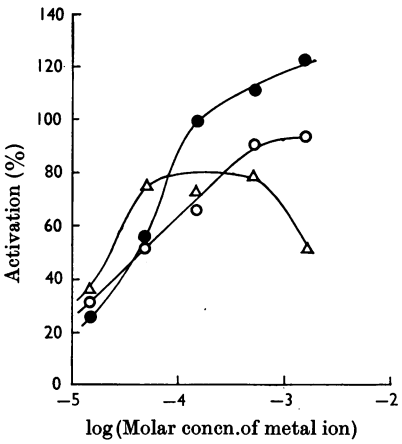

Fig. 1. Effect of increasing concentrations of metal ions on the activation of D-mandelate oxidation. Each sample contained imidazole–HCl buffer, pH7·0 (83mM), D-mandelate (1mM), particulate fraction (167μg. of protein) and soluble fraction (90μg. of protein) in 3ml. Incubation was for 15min. at 21°. ●, In the presence of $MgCl_2$; ○, in the presence of $MnCl_2$; △, in the presence of cobalt acetate.

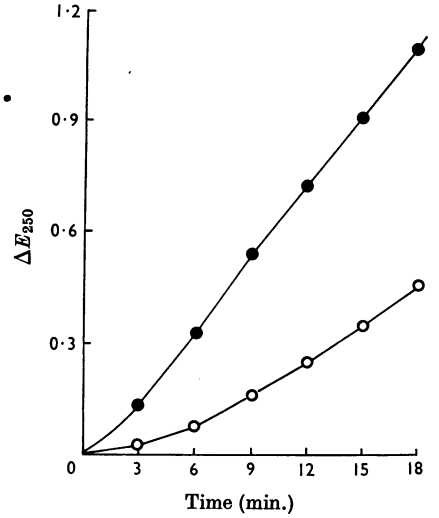

Fig. 2. Oxidation of D-mandelate in the absence and presence of Mg^{2+}. Each sample contained imidazole–HCl buffer, pH7·0 (83mM), D-mandelate (1mM), particulate fraction (167μg. of protein) and soluble fraction (90μg. of protein) in 3ml. ○, No Mg^{2+} added; ●, in the presence of 3mM-$MgCl_2$.

Table 2. *Inhibitory effects of different anions at various concentrations of* Mg^{2+}

Each sample contained imidazole–HCl buffer, pH7·0 (83mM), D-mandelate (1mM), particulate fraction (167μg. of protein) and soluble fraction (90μg. of protein) in 3ml. ΔE_{250} in the control experiment was 1·20 in 18min.

Inhibitor	Concn. (mM)	Time (min.)	Concn. of Mg^{2+} (mM) ...	Change of activity (%) 0·1	0·3	1·0	3·0
NaF	1	6		−34·5	−23·0	−23·0	−31·0
Na_2HPO_4*	83	9		−36·8	−34·6	−38·5	−37·5
$Na_4P_2O_7$*	1	12		−35·6	−25·0	−10·0	−7·1
EDTA*	0·2	9		−52·0	− 3·0	− 1·5	+10·0

* Adjusted to pH7·0.

Table 3. *Inhibitory effects of different anions at various concentrations of* D-*mandelate in the absence of* Mg^{2+}

Each sample contained imidazole–HCl buffer, pH 7·0 (83 mM), and particulate fraction (167 μg. of protein). In Expt. 1 90 μg. and in Expt. 2 135 μg. of soluble-fraction protein was added. The solution of D-mandelate was treated by passage through a column of Dowex A–1 chelating resin. The total volume was 3 ml.

Expt. no.	Inhibitor	Concn. (mM)	Time (min.)	Concn. of D-mandelate (mM).....	Inhibition (%) 0·033	0·10	0·33	1·00	3·33
1	NaF	1	6		60·0	56·0	41·5	24·5	—
	Na_2HPO_4*	83	9		91	75·5	64·3	31·5	—
	$Na_4P_2O_7$*	1	12		78·2	76·5	78·7	—	—
	EDTA*	0·2	18		92	91·8	91·5	—	—
2	$Na_4P_2O_7$*	1	12		—	—	65·4	59·2	65·6
	EDTA*	0·2	18		—	—	80·2	84·6	85·5

* Adjusted to pH 7·0.

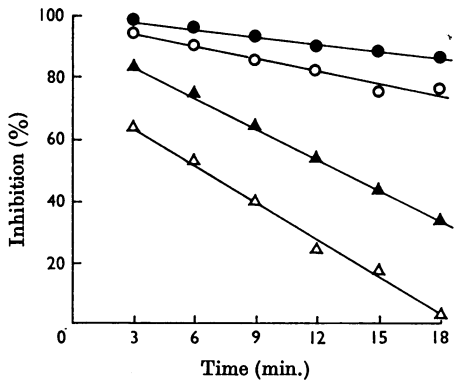

Fig. 3. Decrease of fluoride inhibition during the incubation. Each sample contained imidazole–HCl buffer, pH 7·0 (83 mM), D-mandelate (0·1 mM), particulate fraction (430 μg. of protein) and soluble fraction (148 μg. of protein) in 3 ml. NaF was present in the following final concentrations: ●, 33 mM; ○, 10 mM; ▲, 3·3 mM; △, 1 mM.

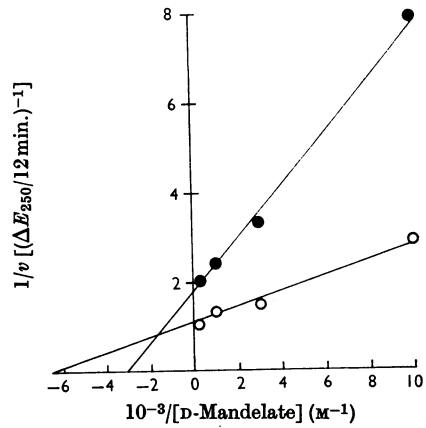

Fig. 4. Oxidation of D-mandelate at various concentrations with and without added Mg^{2+} (Lineweaver–Burk plots). Each sample contained imidazole–HCl buffer, pH 7·0 (83 mM), particulate fraction (167 μg. of protein) and soluble fraction (90 μg. of protein) in 3 ml. Incubation was for 12 min. The curves are the linear regressions calculated by the method of least squares. ●, No Mg^{2+} added; ○, in the presence of 3 mM-$MgCl_2$.

fraction was not affected by 1 mM-EDTA, thus confirming the conclusion that the activity of the dehydrogenase is not enhanced by Mg^{2+} or other metal ions.

The inhibitions by fluoride and phosphate are highest initially and decrease with time. This effect is illustrated in Fig. 3 for fluoride.

The activity of the enzyme system remained unchanged by the addition of thiols (cysteine, glutathione, mercaptoethanol).

Effect of Mg^{2+} *on the Michaelis constant.* As shown by the Lineweaver–Burk plots of Fig. 4, the addition of Mg^{2+} (1 mM) results in a lowering of K_m. In the example shown, K_m, in the absence of Mg^{2+}, was 0·3 mM, and in its presence 0·15 mM. In two other experiments the values for K_m in the presence of Mg^{2+} were 0·12 and 0·21 mM, and in the absence of Mg^{2+} 0·34 and 0·48 mM.

Optimum pH *of racemase and dehydrogenase.* The variation of activity with pH of the two enzymes was determined in 83 mM-succinate–imidazole–glycylglycine buffer (Hagen & D'Iorio, 1965). With D-mandelate and the complete enzyme system, the curve has two peaks at pH 7·0 and 7·8. A two-peaked curve is also obtained when the activity of the dehydrogenase, with L-mandelate as substrate, is measured, but in this case the second peak is

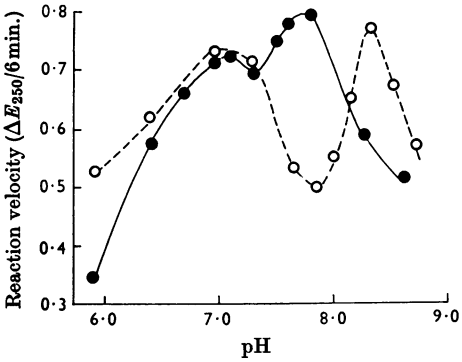

Fig. 5. pH–activity curves for the oxidation of mandelate. Each sample contained succinate–imidazole–glycylglycine buffer (83 mM). Incubation was for 15 min. ●, Oxidation of 1 mM-D-mandelate in the presence of particulate fraction (167 μg. of protein), soluble fraction (45 μg. of protein) and $MgCl_2$ (3 mM) in 3 ml. ○, Oxidation of 1 mM-L-mandelate in the presence of particulate fraction (100 μg. of protein) in 3 ml.

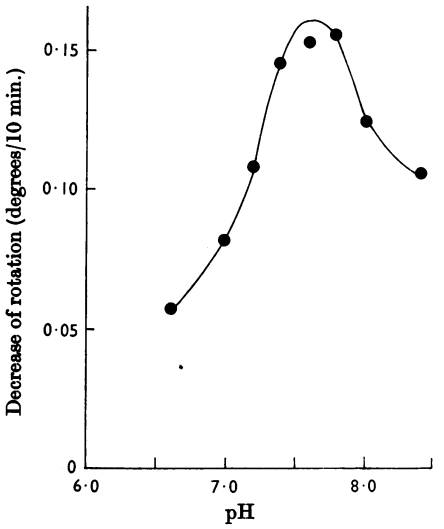

Fig. 6. Optimum pH of mandelate racemase (polarimetric estimation). Each sample contained succinate–imidazole–glycylglycine buffer (83 mM), D-mandelate (10 mM), $MgCl_2$ (3 mM) and soluble fraction (135 μg. of protein) in 3 ml. Incubation was for 10 min.

located at pH 8·2 (Fig. 5). Since the dehydrogenase activity is the result of the action of several enzymes, namely, in addition to the dehydrogenase proper, of the electron-transporting chain and of benzoylformate carboxylase, the presence of more than one pH optimum is understandable. When the two curves were compared, it appeared likely that the pH optimum of the racemase was about 7·8. This was confirmed when the activity of the racemase was studied polarimetrically with D-mandelate as substrate (Fig. 6).

Racemization of the optical isomers. Gunsalus, Stanier & Gunsalus (1953) have shown that both D- and L-mandelate are completely racemized at similar rates. However, when the racemization of the two isomers was observed polarimetrically, it appeared that, at least initially, the D-isomer reacted faster than the L-isomer. For these measurements, 30 μmoles of substrate were incubated at room temperature with 135 μg. of soluble fraction in 3 ml. of 83 mM-succinate–imidazole–glycylglycine buffer, pH 7·8, containing magnesium chloride (3 mM). After 10 min. the rotation of the D-mandelate solution had decreased by 0·156°, that of L-mandelate solution by only 0·046°. After 15 min. the decreases of rotation were 0·204° and 0·090° respectively; thus the reaction was slightly slower for D-mandelate but faster for L-mandelate in the third than in the first two 5 min. intervals. An initial lag period in the racemization of L-mandelate may also be detected in the diagram shown by Gunsalus *et al.* (1953), though the authors did not comment on it. It is unlikely that the slower reaction of L-mandelate can be attributed to the presence of an inhibitory contaminant, since the sample was vigorously oxidized by the particulate fraction.

Substrate specificity of the racemase and dehydrogenase. Three phenolic derivatives of mandelic acid, DL-*p*-hydroxymandelic acid, DL-3,4-dihydroxymandelic acid and DL-4-hydroxy-3-methoxymandelic acid, were tested as substrates for the two enzymes. Two series of experiments were set up in which the substrates were incubated either with the particulate fraction alone or with a mixture of particulate and soluble fractions. In the first series of experiments only the L-isomer was a potential substrate of enzymic oxidation, whereas in the second series both D- and L-isomers were potential substrates, provided that the D-isomer was acted on by the racemase. The substrate concentration was decreased to 0·1 mM so as to make the observation of the end point of the reaction more convenient.

In the absence of the soluble fraction all three phenolic acids were oxidized to their respective aldehydes to the extent of at least a 50% conversion (Fig. 7). The initial speed of the reaction, as a percentage of the rate at which DL-mandelate was oxidized, was 39 for *p*-hydroxymandelate, 27 for 3,4-dihydroxymandelate and 8 for 4-hydroxy-3-methoxymandelate. With *p*-hydroxymandelate and 4-hydroxy-3-methoxymandelate the reaction stopped exactly at the 50% mark. The oxidation

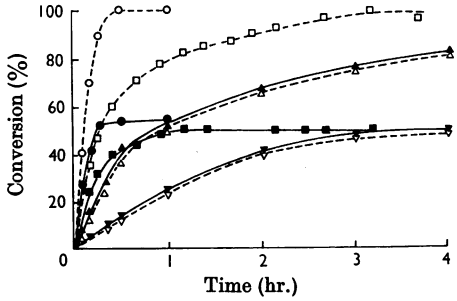

Fig. 7. Oxidation of DL-mandelate and phenolic mandelates. Each sample contained imidazole–HCl buffer, pH 7·0 (83 mM), substrate (0·1 mM), $MgCl_2$ (2 mM) and particulate fraction (333 μg. of protein) in 3 ml. In the experiments represented by the broken lines 180 μg. of soluble-fraction protein was also added. The extinctions corresponding to 100% conversion were calculated from the molecular extinction coefficients of the aldehydes formed as end products. The substrates tested were: ● and ○, DL-mandelate; ■ and □, DL-*p*-hydroxymandelate; ▲ and △, DL-3,4-dihydroxymandelate; ▼ and ▽, DL-4-hydroxy-3-methoxymandelate.

of DL-mandelate slowly continued beyond the 50% mark, presumably because of the presence of traces of racemase in the particulate fraction. The oxidation of 3,4-dihydroxymandelate also continued past the 50% mark at a diminished rate. This was due to non-enzymic oxidation that could be prevented by the addition of EDTA (0·1 mM); however, the addition of EDTA would have inhibited the racemase; in the experiment shown in Fig. 7 this addition was therefore omitted.

When both soluble and particulate fractions were present, DL-mandelate was completely oxidized in 30 min. DL-*p*-Hydroxymandelate was also completely oxidized, but at a much lower rate. The presence of the soluble fraction had no effect whatever on the oxidation of DL-4-hydroxy-3-methoxymandelate or DL-3,4-dihydroxymandelate.

It may be concluded from these results that all three phenolic mandelic acids are substrates of L-mandelate dehydrogenase, although reacting more slowly than L-mandelate. On the other hand, only *p*-hydroxymandelate is attacked by mandelate racemase.

DISCUSSION

The finding that the activity of mandelate racemase is greatly stimulated by the addition of Mg^{2+} and other bivalent metal ions suggests that this enzyme belongs to the group of dissociable metal enzymes, apparently the first case of a racemase thus classifiable. The enzyme possesses activity in the absence of added metal ion activators and some activity was retained even when 1·25 mM-EDTA was substituted for the imidazole buffer in the dialysis step. This may be due to a slow or incomplete dissociation of the natural activator. On the other hand, inhibition was virtually complete when enzyme activity was tested in the presence of low concentrations of EDTA, which strongly suggests that the metal ion requirement is essential for activity. It is now widely assumed (Hellerman, 1937; Smith, 1949; Malmström & Rosenberg, 1959) that the metal ion activator facilitates the formation of the enzyme–substrate complex. The observations presented here support this hypothesis. The Michaelis constant may be described by the kinetic equation:

$$K_m = (k_{-1} + k_{+2})/k_{+1}$$

where k_{+1}, k_{-1} and k_{+2} are the rate constants for the formation of the enzyme–substrate complex and its breakdown either into the starting or the end products of the reaction (cf. Dixon & Webb, 1964). It follows from this equation that K_m is inversely proportional to the velocity of formation of the enzyme–substrate complex. If Mg^{2+} accelerates this process, it would be expected to lower K_m and this is what has been observed. A similar effect of Mg^{2+} has been described by Griffiths, Morrison & Ennor (1957) for ATP–L-arginine phosphotransferase (EC 2.7.3.3).

One of the effects of the metal ion activator is greatly to decrease the initial lag period in the combined enzyme system. Gunsalus *et al.* (1953), who also noticed this initial lag, explained it by the initial absence of oxidizable substrate. If we accept this explanation, then the effect of the metal ion activator is due to the more rapid build-up of saturating concentrations of L-mandelate. However, the existence of a lag phase in the activity of the racemase itself, particularly in the absence of an added metal ion activator, cannot be ruled out. Unfortunately, the polarimetric readings, with the equipment available, were too time-consuming and imprecise to decide this question by direct observation, at any rate with D-mandelate as substrate; with L-mandelate, on the other hand, evidence suggesting the existence of such a lag period was obtained.

The inhibitory effects of EDTA and pyrophosphate, both well-known metal ion chelators, are undoubtedly due to competition with the enzyme for the activator. Phosphate (Greenwald, Redish & Kibrick, 1940) and fluoride, in combination with phosphate (Warburg & Christian, 1942), can also form complexes with Mg^{2+}, but in these cases the inhibition proved to be competitive with respect to the substrate. Nevertheless, it is tempting to speculate that the propensity of these anions to

combine with Mg^{2+} is related to their inhibitory effects, since it might enable them to react with the enzyme–metal ion (enzyme–Mg^{2+}?) complex and thus pre-empt the attachment of the substrate.

After Rosano's (1964) proposal to use L-mandelate dehydrogenase for the estimation of urinary 4-hydroxy-3-methoxymandelic acid, attempts were made to develop a similar method for the estimation of urinary dihydroxymandelic acid (Weil-Malherbe, 1966). However, the results here presented show that the enzymes from *Ps. fluorescens* are not suitable for this purpose. Armstrong, McMillan & Shaw (1957) have demonstrated that urinary 4-hydroxy-3-methoxymandelic acid has the D-configuration, and the same is presumably true for urinary 3,4-dihydroxymandelic acid. Any oxidation of these compounds would therefore depend on a prior conversion of the D- into the L-isomer. But whereas this conversion is readily brought about by mandelate racemase with D-mandelate and, less readily, with D-*p*-hydroxymandelate, neither D-4-hydroxy-3-methoxymandelic acid nor D-3,4-dihydroxymandelic acid is attacked by the enzyme. The good agreement between the chemical and enzymic assay of urinary 4-hydroxy-3-methoxymandelic acid reported by Rosano (1964) is difficult to understand in the light of these findings.

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