Stereospecific Enzymes in the Degradation of Aromatic Compounds by *Pseudomonas putida*

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Two reactions in the catabolism of catechol by *meta*-fission, namely, hydration of 2-oxopent-4-enoate (vinylpyruvate) and aldol fission of the product, are catalyzed by stereospecific enzymes. The absolute configuration of this hydration product was shown to be L-(S)-4-hydroxy-2-oxopentanoate. Vinylpyruvate hydratase, purified almost to homogeneity, had a molecular weight of about 287,000 and was dissociated in sodium dodecyl sulfate, without prior treatment with mercaptoethanol, into a species with an approximate molecular weight of 28,000. The hydratase was highly specific for its substrates; thus, although 2-oxo-*cis*-hex-4-enoate was also hydrated, structurally similar compounds such as the *trans* isomer, vinylacetic and crotonic acids, and the ring-fission products of catechol and methylcatechols were not attacked. Vinylpyruvate hydratase was activated by Mn^{2+} ions. On the basis of these observations, a mechanism is proposed which closely resembles that for 4-hydroxy-2-oxopentanoate aldolase. A possible evolutionary connection between functionally related, divalent cation-activated hydro-lyases and aldolases is discussed. It was also demonstrated that L-(S)-4-hydroxy-2-oxohexanoate is the biologically active enantiomer of this hydroxy acid.

It is generally accepted that a prerequisite for enzymatic fission of the benzene ring, and hence its utilization for growth by microorganisms, is the insertion of two hydroxyl groups into the nucleus. Thus, various species of *Pseudomonas* convert benzene (11, 13) or phenol (7) into catechol; 3-methylcatechol is given from toluene (12, 14) and from *o*- and *m*-cresols (2); and *p*-cresol (2) gives 4-methylcatechol. Other pseudomonads employ alternative pathways in which methyl group substituents are first oxidized (reviewed in 5), but two hydroxyl groups are subsequently inserted to form, for example, gentisic and protocatechuic acids as the substrates for ring-fission dioxygenases. The pathways of degradation of catechol and protocatechuate by *ortho*-fission have been thoroughly elucidated (22-24). The present state of our knowledge of the *meta*-fission pathway for catechol is shown in Fig. 1, where 2-hydroxymuconic semialdehyde (I) is formed by the action of catechol 2,3-oxygenase (EC 1.13.1.2). Alternative reactions for I may then occur, namely, hydrolytic fission (a) or oxidation (b) to give oxalocrotonic acid (II). In *P. putida*, the enzymes of Fig. 1 are able to metabolize methylcatechols as well as catechol; however, 3-methylcatechol is degraded by

route a exclusively, hydrolysis liberating acetate instead of formate, because oxidation is prevented when the substrate contains methyl at C-6 instead of hydrogen. By contrast, route b predominates when pseudomonads metabolize catechol and 4-methylcatechol (3, 26), and also operates almost exclusively for catechol degradation in benzoate-grown *Azotobacter* (25). The two pathways converge to give the common metabolite 2-oxopent-4-enoic acid (III) when catechol and 3-methylcatechol are degraded (1), or to give 2-oxohex-4-enoic acid in the case of 4-methylcatechol, since the methyl substituent is then retained (1). The next reaction in the sequence is a hydration which furnishes 4-hydroxy-2-oxopentanoate (IV) or, in the case of 2-oxohex-4-enoic acid, 4-hydroxy-2-oxohexanoate. The hydroxyoxo acids then undergo aldol fission to pyruvate, the former giving, in addition, acetaldehyde as shown in Fig. 1, and the latter, propionaldehyde.

The present work is concerned with stereochemical aspects of the hydration of III and . aldol fission of IV, and with the corresponding reactions of their homologues bearing methyl groups at C-5. Compound IV possesses an asymmetric carbon atom (C-4), and the sug-

gestion has been made (7) that, since cell-free extracts attack about half of the chemically synthesized compound, only one of the two enantiomers is biologically active. This has been confirmed, and we have established the absolute configuration of this enantiomer.

MATERIALS AND METHODS

Partial purification of cell extracts. The organism used was isolated in 1963 from mud in a creek in Urbana, Ill., and is designated *Pseudomonas* U. It was later deposited with the British National Collection of Industrial Bacteria, identified as *Pseudomonas putida*, and assigned the collection number NCIB 10015. Dagley and Gibson (7) have described its maintenance and growth with 0.03% phenol, the carbon source used throughout this work, and also the preparation of crude cell-free extracts. By simple fractionation procedures, Bayly and Dagley (1) obtained two types of preparation: one that converted catechol, or methylcatechols, into their ring-fission products, and the other that accumulated hydroxyoxo acids from these substrates. They referred to these preparations as "heat-treated extract" and "Sephadex-treated extract," respectively, and their designations are retained in the present communication. Crude extracts contained an aldolase which cleaved IV (Fig. 1) and also its homologue, 4-hydroxy-2-oxohexanoate, but did not attack 4-hydroxy-4-methyl-2-oxoglutarate or citramalate, which are substrates for other aldolases serving protocatechuate (6) and gentisate (17) pathways, respectively. The aldolase was assayed spectrophotometrically by determining the pyruvate formed, but this could not be done directly since extracts catalyzed oxidation of reduced nicotinamide adenine dinucleotide (NADH) before the addition of lactate dehydrogenase. The assay mixture contained, in a total volume of 4.65 ml, 140 µmoles of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.5, 12 µmoles of MgCl₂, 6 µmoles of DL-4-hydroxy-2-oxopentanoate, 6 µmoles of mercaptoethanol, and 0.05 ml of enzyme. The mixture was incubated at 30 C, and samples of 1 ml,

withdrawn at intervals up to 15 min, were held at 100 C for 3 min and then cooled and filtered through glass wool. Pyruvate was determined from the increase in absorbance at 340 nm which was observed when 0.4 ml of the filtered solution was added to 1.0 ml of 0.05 M Tris-hydrochloride buffer, pH 8.5, containing 0.3 mg of NADH and 5 µliters (17 units) of lactate dehydrogenase. A plot of pyruvate formed, against time of incubation, was linear over 15 min. An aldolase preparation, purified threefold from crude extract and having a specific activity of 0.04 µmole of pyruvate per min per mg of protein, was obtained by bringing crude extract to 40% saturation with solid (NH₄)₂SO₄. The precipitate was taken up in 0.05 M Tris-hydrochloride buffer, dialyzed against the same buffer, and applied to a column of diethylaminoethyl (DEAE)-cellulose (40 by 1.5 cm). Protein was eluted with 600 ml of a linear gradient from 0.1 to 0.2 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 8.5, and 10-ml fractions were collected. Enzymatic activity was located in fractions 22 to 28, which were pooled, reduced in volume by pressure dialysis to about 10 ml, and stored at −15 C. The activity of the partially purified aldolase, which contained 5 mg of protein/ml, was retained on freezing.

Preparation of substrates. The synthesis of 4-methyl- and 4-ethyl-2-oxobutyrolactone, and their hydrolysis to give solutions of 4-hydroxy-2-oxopentanoate and 4-hydroxy-2-oxohexanoate, respectively, were as previously described (2). The synthesis of 2-oxopent-4-enoic acid (vinylpyruvate; III, Fig. 1) has not been described, but Coulter and Talalay (4) have prepared 2-oxohex-4-enoic acid (II, Fig. 7) by both chemical and enzymatic methods. We used a similar enzymatic synthesis, except that L-amino acid oxidase was used instead of D-amino acid oxidase (4) for converting the amino acid into its keto acid. DL-Allylglycine (60 µmoles) was added to a reaction mixture which contained, in a total volume of 6 ml, 200 µmoles of KCl, 140 µmoles of Tris buffer (pH 7.2), 0.5 ml of L-amino acid oxidase (10 µliters of the commercial enzyme diluted with 0.5 ml of 0.1 M NaCl) and 0.1 ml of catalase. After incubating at 37 C for 1 hr with shaking, 1 ml of 6% perchloric acid was added, and the precipitate was allowed to settle
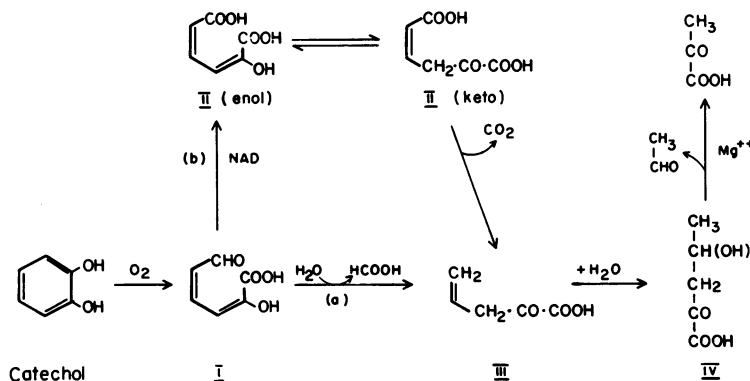

FIG. 1. *Pathways for the metabolism of catechol in species of Pseudomonas.*

and then removed. The clear solution was extracted with 3 volumes of ether, the ethereal solution was dried over Na_2SO_4, and the solvent was carefully evaporated in a stream of air to leave a volatile oil which had an odor similar to that of vinylacetic and crotonic acids. The oil was readily soluble in water and gave a strong blue color with a solution of $FeCl_3$, indicative of an enolic group. The ultraviolet spectrum of the aqueous solution showed a λ_{max} at 265 nm, which shifted to 270 nm on acidification and to 305 nm on adding dilute base. For compound II (Fig. 7) prepared enzymatically, Coulter and Talalay (4) reported values of 274, 281, and 307 nm, respectively. We also found that the absorption in base was transitory, presumably because the enolate ion is chemically unstable (4). In addition to these observations, the structure assigned, namely, that of III (Fig. 1), was confirmed by converting the material into norvaline and vinylacetic acid, respectively. Norvaline was formed by reduction of the 2,4-dinitrophenylhydrazone of III as described by Bayly and Dagley (1) and was also identified by the methods they used. Vinylacetic acid was formed by treating the solution with 4% H_2O_2, removing excess of reagent with catalase, and extracting with ethyl acetate. This extract was dried with Na_2SO_4 and a portion was examined by gas-liquid chromatography on a diethylene glycol-succinate column at 150 C; two peaks were observed, one of which was enriched by addition of authentic vinylacetic acid and the other by crotonic acid. The appearance of crotonic acid may be ascribed either to a shift of the double bond of vinyl pyruvate at C-5 and C-4 to a new position between C-4 and C-3 during oxidative decarboxylation by H_2O_2 or to a similar change in vinylacetic acid; such shifts are well documented for compounds of this type, particularly in alkaline solution (15).

For experiments with vinylpyruvate as a substrate, it was convenient to use deproteinized solutions from the deamination of allylglycine, rather than attempt to work with the isolated compound which, like 2-oxohex-4-enoic acid (4), is rather unstable even under N_2 at -20 C. The acidified solutions, however, were relatively stable if kept at 4 C; at this temperature, there was a slow conversion (about 50% in 2 weeks) to 4-methyl-2-oxobutyrolactone (1), and these solutions were therefore not brought to the pH of the reaction under investigation until ready for use. Vinylpyruvate in these solutions was converted into pyruvate and acetaldehyde at pH 7.2 by a dialyzed crude extract. The products were identified as their 2,4-dinitrophenylhydrazones (7). A Sephadex-treated extract accumulated 4-hydroxy-2-oxopentanoate which was similarly identified, both as acid and as lactone (2).

Solutions of cis and trans forms of 2-oxohex-4-enoic acid were prepared, as described for vinylpyruvate, by oxidative deamination of the corresponding amino acids (cis- and trans-crotylglycine); these were the kind gifts of W. Shive of the University of Texas at Austin. Ultraviolet absorption maxima for 2-oxo-cis-hex-4-enoic acid were observed at the wavelengths reported by Coulter and Talalay (4); those for

the trans compound were at 270, 275, and 302 nm, respectively, when in neutral, acidic, and basic solutions.

Oxidative decarboxylation of 4-hydroxy-2-oxopentanoate. Proof that only one enantiomer of 4-hydroxy-2-oxopentanoate is biologically active rested upon the observation that the racemate is quantitatively oxidized to 3-hydroxybutyric acid by hydrogen peroxide. This product was identified in preliminary experiments as follows. To 100 ml of 2 mM DL-4-hydroxy-2-oxopentanoate in 0.01 M phosphate buffer, pH 7.2, were added 8 ml of 3% hydrogen peroxide; after incubating for 30 min at 30 C, excess peroxide was removed by catalase. Tests for a carbonyl function were then negative, but on treatment with concentrated H_2SO_4 a portion of the solution gave the ultraviolet spectrum of crotonic acid, a compound known to be formed from 3-hydroxybutyrate by dehydration (29). The remainder of the solution was acidified; it was then taken into ether by continuous extraction, and the volume was reduced by evaporation. When chromatographed on a thin layer of Kieselgel G (2) with benzene-methanol-acetic acid (45:8:4, v/v) as solvent, one acidic spot with the same R_F (0.63) as authentic 3-hydroxybutyrate was detected when sprayed with bromocresol green. The bulk of the ether extract was applied to a column (30 cm) of Celite, prepared in a 50-ml burette, and was eluted as described by Swim and Utter (30). One acidic component was collected in the same fractions as those for authentic 3-hydroxybutyrate. This compound was assayed by the method of Williamson, Mellanby, and Krebs (33), in which hydrazine is used to drive to completion the NAD-linked enzymatic oxidation of the D enantiomer.

Purification of VP hydratase. The present investigation has established the configuration of IV (Fig. 1), and the systematic name for the enzyme that converts III into IV may therefore be assigned, namely, L-(S)-4-hydroxy-2-oxopentanoate hydrolyase. However, since we have no evidence for reversibility, and the reaction functions solely for hydration, we shall refer to the enzyme by the abbreviated name of vinylpyruvate hydratase (VP hydratase). Development of a spectrophotometric assay based on the disappearance of vinylpyruvate was hindered by the fact that absorbance at 265 nm, although stable in acid, decreased spontaneously at pH 7.2. Since ultraviolet light absorption by 2-oxohex-4-enoic acid is due to the dienol form (4), it is feasible that the spontaneous decrease may be due to tautomeric change. However, a reliable assay was provided by the following procedure. Sufficient vinylpyruvate was added to the reaction mixture (total volume, 3 ml) in 0.05 M phosphate buffer of pH 7.2 containing 0.33 mM $MnCl_2$ to provide an initial absorbance of 2; this amount of substrate was present in about 0.4 ml of a solution freshly prepared from DL-allylglycine as described. The absorbance was allowed to decrease by 0.2, at which time the enzyme to be assayed was added, giving an increased rate of decline (Fig. 2). Calculations of enzyme activity were always made for amounts of enzyme that produced a rate of decrease
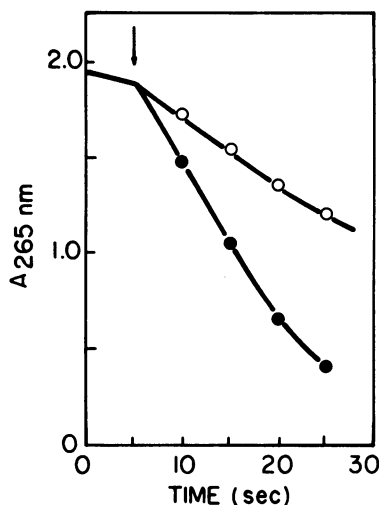
FIG. 2. *Spectrophotometric assay of vinylpyruvate hydratase, as described in Materials and Methods. At the point indicated by the arrow, cell extract was added: 5 μliters, O; 10 μliters, ●.*

in absorbance at least five times greater than the spontaneous rate of decrease. In describing the purification procedure (Table 1), the unit of activity is based directly upon spectrophotometric measurements and is defined as that amount of enzyme which catalyzes a decrease of 1 unit of absorbance per min. In water, 2-oxohex-4-enoic acid has $\epsilon = 10,000$ (4), so that activity in absorbance units could be expressed as micromoles of vinylpyruvate per minute simply by dividing by 10, if vinylpyruvate absorbs light in the same fashion. Measurements of yields of pyruvate formed, when vinylpyruvate was degraded by dialyzed crude extracts, were about 80% of those calculated by assuming $\epsilon = 10,000$.

The starting material for the purification of VP hydratase was a batch of 250 g (wet weight) of *P. putida* cells grown with phenol. Cells were grown in 100 liters of medium in a stainless-steel fermentor provided with stirring and forced aeration. After inoculation with 1 liter of an overnight culture, growth proceeded for 10 hr, and an additional 30 g of phenol was then added. Cells were harvested at maximal turbidity and broken by sonic treatment; an extract was prepared in 0.05 M phosphate buffer, pH 7.2 (7). The crude extract was heat-treated (1), and the clear supernatant fluid was brought to 40% saturation with solid $(NH_4)_2SO_4$. The precipitate was taken up in 0.05 M Tris-hydrochloride buffer, pH 8.5, dialyzed against the same buffer until free from ammonium ions, and then applied to a DEAE-cellulose column (25 by 4.5 cm) which had equilibrated with this buffer. The column was eluted with 1 liter of a linear gradient of 0.15 to 0.30 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 8.5, and 130 fractions of 7 ml were collected. Protein, monitored by measurement of absorbance at 280 nm, appeared in all fractions, but VP hydratase was present only in tubes

107 to 125. The active fractions were pooled and brought to 70% saturation with $(NH_4)_2SO_4$; the precipitate was taken up in 0.05 M Tris buffer, pH 8.5, containing 0.1 M NaCl. After dialysis, the extract was applied to a column of Sephadex G-200, and the enzyme was eluted with NaCl-Tris buffer solution. This step, which was included with the object of achieving a separation based on molecular size, did not prove profitable as no significant increase in specific activity was achieved (Table 1). Active fractions were pooled; protein was concentrated by pressure dialysis and then applied to a triethylaminoethyl-cellulose column (32 by 2 cm) equilibrated with Tris buffer. The column was eluted with 600 ml of a linear concentration gradient of 0.1 to 0.5 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 8.5; fractions of 5 ml were collected, and activity was confined to tubes 40 to 56. Each of these fractions was examined by analytical disc gel electrophoresis with 10% polyacrylamide gels at pH 8.9 according to the procedure of Davis (8). Fractions showing essentially one band were pooled, concentrated, and then dialyzed against 0.1 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 8.5. The purification of the protein, which contained trace amounts of two contaminants as indicated by electrophoresis, was 28-fold and is summarized in Table 1. The low yield of 1% was due, in part, to a very critical selection of fractions in the last step, yielding a small sample of enzyme for the present investigation which was as pure as possible.

Physicochemical examination of purified VP hydratase. Sedimentation velocity experiments were performed in a Spinco model E analytical ultracentrifuge with the use of schlieren optics according to the procedure of Schachman (27). Having determined the value of $S_{20,w}^{0}$ for the enzyme, and taking $\bar{v} = 0.73$, one can calculate the molecular weight from the Svedberg equation (27) if the diffusion coefficient is known. This was obtained by measurement of the spreading of a concentration gradient

TABLE 1. *Purification of vinylpyruvate hydratase*

Step	Protein (mg)	Specific activity[a]	Yield (%)
Crude extract	22,000	360	100
Heat treatment	11,800	620	95
Ammonium sulfate precipitation	3,400	1,320	56
Diethylaminoethyl - cellulose chromatography	640	1,800	15
Sephadex G-200 chromatography	315	1,800	7
Triethylaminoethyl - cellulose chromatography	7.2	10,000	1

[a] Specific activity is expressed as units per milligram of protein. A unit is defined as the amount of enzyme that catalyzes a decrease of 1 unit of absorbance at 265 nm per min; see Materials and Methods for details.

with time, by use of a double-sector synthetic-boundary cell. The boundary was observed at intervals of time with schlieren optics at a rotor speed of 15,200 rev/min, and the diffusion coefficient was determined from a plot of A^2/H^2 against time, where A and H, respectively, are the area and height of a peak (21).

Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Weber and Osborn (32); gels were stained for protein with Coomassie brilliant blue (Sigma C-8127) for 16 hr and were destained in 18% methanol-9% acetic acid. To determine whether subunits of VP hydratase were joined by disulfide bridges which could be cleaved by reduction, we prepared reduced and nonreduced samples of enzyme. The enzyme was brought to a concentration of 1 μg of protein/μliter of 1 mM phosphate buffer, pH 7.1, containing 1% SDS. This solution was divided and to one-half β-mercaptoethanol was added to a final concentration of 10%. Both samples were incubated at 90 C for 20 min, and 10 to 15 μliters of 0.05% bromophenol blue was added to each with 1 drop of glycerol; 10 μg of protein was then applied to each gel.

Materials. Vinylacetic acid and crotonic acid were from Aldrich Chemical Co., Milwaukee, Wis.; DL-allylglycine, from Nutritional Biochemicals Corp., Cleveland, Ohio; DL-3-hydroxybutyrate (sodium salt), D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), L-amino acid oxidase (EC 1.4.3.2), and catalase (EC 1.11.1.6) were from Sigma Chemical Co., St. Louis, Mo.; and lactate dehydrogenase (EC 1.1.1.27) was from Calbiochem, Los Angeles, Calif.

RESULTS

Degradation of DL-4-hydroxy-2-oxopentanoate with partially purified aldolase. Preliminary determinations were made, by use of the method of Friedemann and Haugen (10), of the amounts of pyruvate formed when a synthetic sample of 4-hydroxy-2-oxopentanoate was degraded by partially purified aldolase. The results were in agreement with earlier observations (7) that only one-half of the sample was decomposed. However, when pyruvate was determined by use of lactate dehydrogenase, 1 μmole of NADH was oxidized for 1 μmole of racemic mixture. This discrepancy was resolved when it was shown that NADH was oxidized by the products of aldol fission, even when lactate dehydrogenase was omitted from the reaction mixture. The aldolase preparation was found to contain alcohol dehydrogenase (EC 1.1.1.1), so that acetaldehyde, which is formed in addition to pyruvate, was reduced when NADH was present. Oxidation of NADH by acetaldehyde, formed by aldol fission of 0.45 μmole of DL-4-hydroxy-2-oxopentanoate, was observed at 340 nm for 18 min (Fig. 3). The rate of decrease in absorbance had then attained

about the same value as that for NADH and aldolase alone. When this endogenous oxidation was allowed for, the changes in absorbance indicated that 0.20 μmole of acetaldehyde had been formed, and also that the total amount of pyruvate and acetaldehyde, determined with lactate dehydrogenase present, was 0.37 μmole. From these results, it appeared that 80 to 90% of one enantiomer of the substrate was degraded in 18 min, leaving the second enantiomer (0.225 μmole) unchanged. The remaining 4-hydroxy-2-oxopentanoate was investigated by incubating each reaction mixture with 0.1 ml of 1% hydrogen peroxide at 25 C for 3 min to form 3-hydroxybutyrate. Catalase (10 μliters, 1,500 units) was added and incubation was continued for 1 min; the mixture was then held at 100 C for 3 min, after which it was cooled and samples of 1 ml were assayed for D-3-hydroxybutyrate (33). In Fig. 4, it can be seen that each sample gave essentially the same result, indicating that about 0.2 μmole of the D-enantiomer was formed from the 0.45 μmole of DL-4-hydroxy-2-oxopentanoate originally taken. These experiments were repeated for DL-4-hydroxy-2-oxohexanoate; alcohol dehydrogenase and D-3-hydroxybutyrate dehydrogenase are able to attack propionaldehyde and D-3-hydroxyvalerate respectively, though rather more slowly than the substrates used previously (20). The results showed that only L-4-hydroxy-2-oxohexanoate was attacked by the aldolase: the D enantiomer remained and was oxidized to D-3-hydroxyvalerate on addition of hydrogen peroxide.

These findings were confirmed by converting catechol enzymatically into 4-hydroxy-2-oxopentanoate, which, after oxidation by hydrogen peroxide, yielded a sample of 3-hydroxybutyrate that did not serve as substrate for D-3-hydroxybutyrate dehydrogenase. A reaction mixture contained, in 7 ml, 25 μmoles of 0.05 M phosphate buffer, pH 7.4, and Sephadex-treated extract (12 mg of protein). The solution was shaken at 30 C and 10 μmoles of catechol was added in small amounts over a period of 1.5 hr. At this time, 4-hydroxy-2-oxovalerate was shown to have accumulated by heating a portion of the acidified solution to form the lactone (2, 7) which has characteristic ultraviolet absorption maxima in acid and base. To 1 ml of the remainder of the solution was added 0.1 ml of 3% hydrogen peroxide, and after incubating for 10 min at 30 C any peroxide that remained was removed by adding 10 μliters of catalase. After this treatment, 4-hydroxy-2-oxopentanoate could not be de-
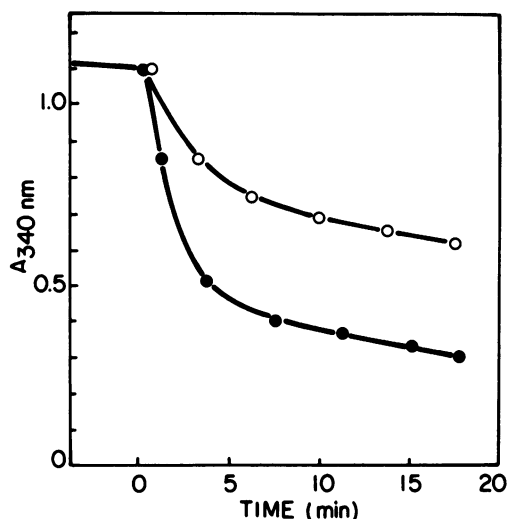
FIG. 3. *Degradation of DL-4-hydroxy-2-oxopentanoate by partially purified aldolase. Cuvettes contained, in 3 ml, 100 μmoles of Tris buffer, pH 8.4, 2 μmoles of MgCl₂, 0.45 μmole of DL-4-hydroxy-2-oxopentanoate, and 0.6 μmole of NADH. Reactions were started by adding 0.6 mg of aldolase protein. Lactate dehydrogenase (34 units) added, ●; no addition, ○.*

tected, but 3-hydroxybutyrate was readily demonstrated as described in Materials and Methods. However, since this sample of the compound did not serve as substrate for D-3-hydroxybutyrate dehydrogenase it was assumed to be the L enantiomer.

Properties of VP hydratase. Although the enzyme, purified as in Materials and Methods, hydrated vinylpyruvate in the absence of added cofactors, activity was stimulated fivefold by 0.3 mM MnCl₂; no stimulation was observed for Mg^{2+}, Zn^{2+}, or Fe^{2+}. When sedimented in an analytical ultracentrifuge, one main peak was given, with indications of small amounts of contaminating material. Sedimentation coefficients for the enzyme, measured at concentrations of 1.2, 4.8, and 10.0 mg/ml were, respectively, 11.5×10^{-13}, 10.5×10^{-13}, and 9.8 $\times 10^{-13}$ sec, and the linear plot of reciprocals, when extrapolated to zero concentration, gave $S_{20,w}^{0} = 11.9 \times 10^{-13}$ sec. The diffusion coefficient was determined at one concentration, as described in Materials and Methods, and gave $D_{20,w} = 3.75 \times 10^{-7}$ cm² sec⁻¹. From these values, an approximate molecular weight of 287,000 was calculated for the enzyme. When submitted to gel electrophoresis in the presence of SDS, the denatured protein had mobilities of 0.60 and 0.63 before and after treatment with

mercaptoethanol, respectively. Bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* (Fig. 5) were also run as standards, and, from the linear plot of electrophoretic mobilities against the logarithms of the known molecular weights, that of the subunit was found to be 28,000. Reduction with mercaptoethanol might have released some material, since the band due to a contaminant of lower molecular weight appeared to be strengthened. However, the mobility of the main band due to the material treated with mercaptoethanol gave a molecular weight of 27,600, which agrees within experimental error (9) with the suggestion that the native enzyme contains 10 subunits.

The enzyme exhibited a narrow substrate specificity. Although 2-oxo-*cis*-hex-4-enoic acid (II, Fig. 7) was hydrated as rapidly as vinylpyruvate, shown by a rapid decrease in light absorbance at 274 nm, the *trans* isomer was not attacked. The product of hydration of the *cis* isomer was shown to be 4-hydroxy-2-oxohexanoate as follows. VP hydratase (2 ml) from the DEAE-cellulose step of purification was incubated at 30 C for 30 min with 60 ml of phosphate buffer, *p*H 7.2, 0.3 mM MnCl₂, and 2 ml of a solution of 2-oxo-*cis*-hex-4-enoic acid prepared by enzymatic deamination of *cis*-
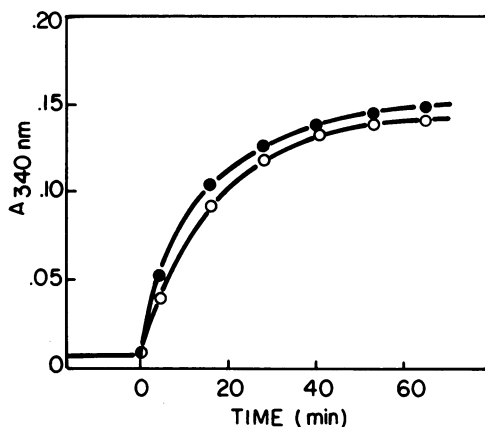

FIG. 4. *Determination of D-3-hydroxybutyrate formed by degrading DL-4-hydroxy-2-oxopentanoate. After performing the experiment of Fig. 3, the hydroxyoxopentanoate which remained was converted into 3-hydroxybutyrate by treatment with hydrogen peroxide. A sample of solution (1 ml) was then incubated with D-3-hydroxybutyrate dehydrogenase, and the reduction of NAD was observed at 340 nm. The two curves refer to the presence (●) and absence (○) of lactate dehydrogenase in the original reaction mixtures which had been incubated with the aldolase.*
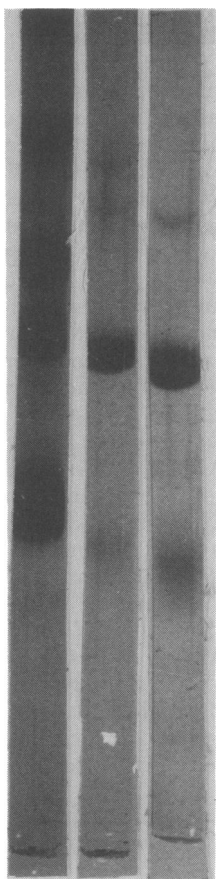
substituents at C-5, respectively, were not hydrated; neither was the product from 4-methylcatechol (Fig. 7, compound I). This was proved by the following experiment. Solutions of ring-fission products in 0.05 M phosphate buffer, pH 7.2, were prepared from catechol, 3-methylcatechol, and 4-methylcatechol by the action of a heat-treated extract of *P. putida* as described by Bayly and Dagley (1). When purified hydratase was added, no reaction took place; but on adding crude extract, which contains a hydrolase for each compound, there was an immediate decrease in absorbance (Fig. 6). It was also shown that the purified enzyme did not hydrate vinylacetic, crotonic, or fumaric acids.

DISCUSSION

Stereospecific enzymatic hydration, followed by aldolase fission of one enantiomer of the hydroxy acid so formed, has now been demonstrated for three pathways of aromatic catabo-
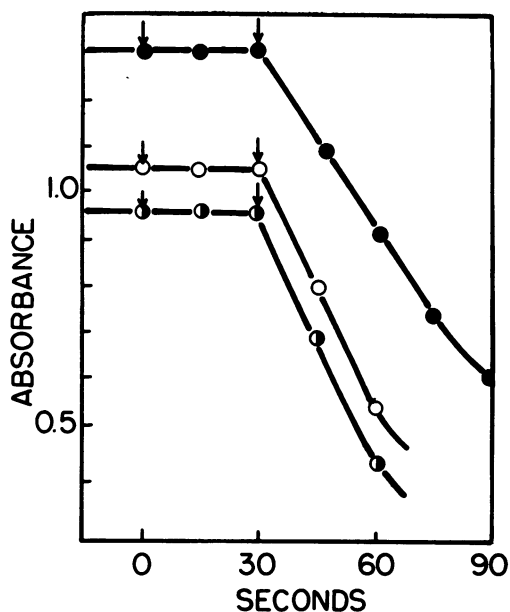

FIG. 5. *Electrophoresis of vinylpyruvate hydratase on SDS poly-acrylamide gels. At the left are shown reference proteins: (top to bottom) bovine serum albumin, chymotrypsinogen, ovalbumin, and cytochrome c. Vinylpyruvate hydratase is shown before (center) and after (right) treatment with mercaptoethanol.*

crotylglycine. The reaction mixture was deproteinized with HCl and divided into two halves, one of which was kept at 4 C while the other was held at 100 C for 5 min to form the lactone of the hydroxyoxo acid. Dinitrophenylhydrazones were formed, extracted into ethyl acetate, and examined as described by Bayly et al. (2) with the use of their solvents B and C. The solution which had not been heated gave a strong spot for the derivative of 4-hydroxy-2-oxohexanoate and a weak spot for unchanged 2-oxo-*cis*-hex-4-enoate, whereas the heated solution gave only the dinitrophenylhydrazone of 4-ethyl-2-oxobutyrolactone.

The ring fission products from catechol and 3-methylcatechol, which contain the vinylpyruvate structure with formyl and acetyl

FIG. 6. *Incubation of cell extract, or partially purified vinylpyruvate hydratase, with ring-fission products of catechol and methylcatechols. Cuvettes contained, in 3 ml, phosphate buffer of pH 7.2 and sufficient ring-fission product to give the initial absorbances shown when measured at maxima for: catechol product, 375 nm (O); 3 methylcatechol product, 388 nm (◑); 4-methylcatechol product, 382 nm (●). Additions of vinylpyruvate hydratase (4 mg of protein) from the DEAE-cellulose step of purification were made at zero time, and at 30 sec 3.9 mg of crude cell extract protein was added to each cuvette.*

lism. Thus, in the degradation of methylgentisic acids by a species of *Pseudomonas*, citraconic acid is formed and then hydrated to give D-citramalic acid; this compound undergoes fission by a coenzyme A-dependent aldolase, specific for the D isomer (17). In the *meta*-fission pathway for protocatechuate, 4-hydroxy-4-methyl-2-oxoglutarate is given by hydration (6) and is then cleaved by a Mg^{2+}-dependent aldolase which is specific for one enantiomer (31). The present work shows that, in the *meta*-fission pathway for catechol and methylcatechols, 2-oxo-pent-4-enoic acid (vinylpyruvate) and 2-oxo-*cis*-hex-4-enoic acid are hydrated to give, respectively, L-(S)-4-hydroxy-2-oxopentanoate (IV, Fig. 7) and L-(S)-4-hydroxy-2-oxohexanoate (III). Although several structurally related compounds were tried as substrates, these two were the only hydrations catalyzed. In particular, the fact that vinylacetic and crotonic acids were not hydrated indicates that an enolizable carbonyl group must be present in substrates for VP hydratase. This structural requirement, together with the stimulation of enzyme activity produced by Mn^{2+} ions, forms the basis for the mechanism proposed in Fig. 8. The redistributions of electrons, occurring during hydration, allow first a proton and then a hydroxyl group to be added to vinyl pyruvate (I), and these shifts are facilitated by alterations in the charge on the coenzyme. This mechanism closely resembles that proposed for the fission of compound II which is catalyzed by a Mg^{2+}-dependent aldolase (Fig. 8). It is also probable, for the following reasons, that similar shifts of electrons occur when compound II is formed by

enzymatic hydrolysis of 2-hydroxymuconic semialdehyde (reaction a of Fig. 1). Consider the hydrolysis of methyl-substituted 2-hydroxymuconic semialdehyde (I, Fig. 7). This reaction is accompanied by a simultaneous change in structure, from *trans* to *cis*, which is required to produce the correct stereoisomer (II, Fig. 7) as substrate for VP hydratase. Thus, we found that the *trans* isomer of II was not hydrated; Coulter and Talalay (4) also established that II (Fig. 7) was attacked stereospecifically by extracts of steroid-induced *P. testosteroni*. This change of configuration was readily explained (28) by movements of electrons that were due, like those of Fig. 8, to the interconversion of keto and enol forms. During the course of these reactions, a transitory intermediate was formed which no longer possessed a double bond between C-4 and C-5, so that rotation of the methyl group to a *cis* position was thereby permitted.

These observations may be related to theories concerning the evolution of catabolic pathways. The step-wise retrograde scheme of Horowitz (18, 19), when combined with the concept of tandem gene duplication, might account for the origins of biosynthetic pathways in bacteria, but specific problems arise when the theory is applied to catabolism. Thus, as Wu, Lin, and Tanaka (34) have noted, successive enzymes in catabolism often catalyze very dissimilar reactions, so that it would appear unlikely that their genes would arise from one another. However, although the *meta*-fission pathway employs enzymes of various types, catalyzing hydrations as well as aldol and hydrolytic fissions, the mechanisms of enzyme
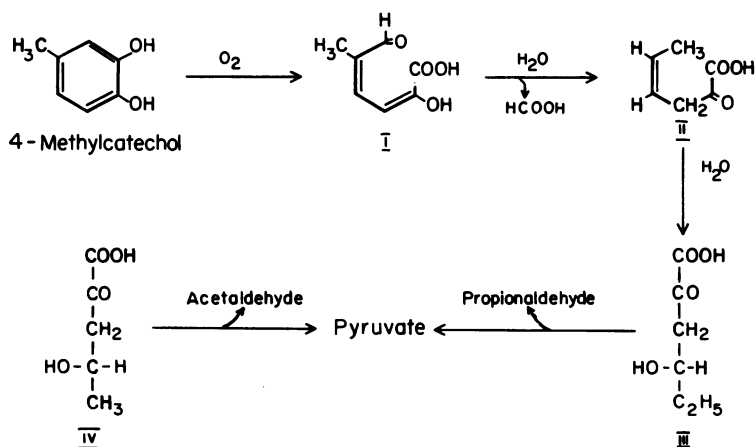

FIG. 7. *Degradation of 4-methylcatechol to give* L-(S)-4-hydroxy-2-oxohexanoate (III). *Catechol and 3-methylcatechol give rise to* L-(S)-4-hydroxy-2-oxopentanoate (IV). *An aldolase cleaves either III or IV to give pyruvate plus propionaldehyde or acetaldehyde, respectively.*
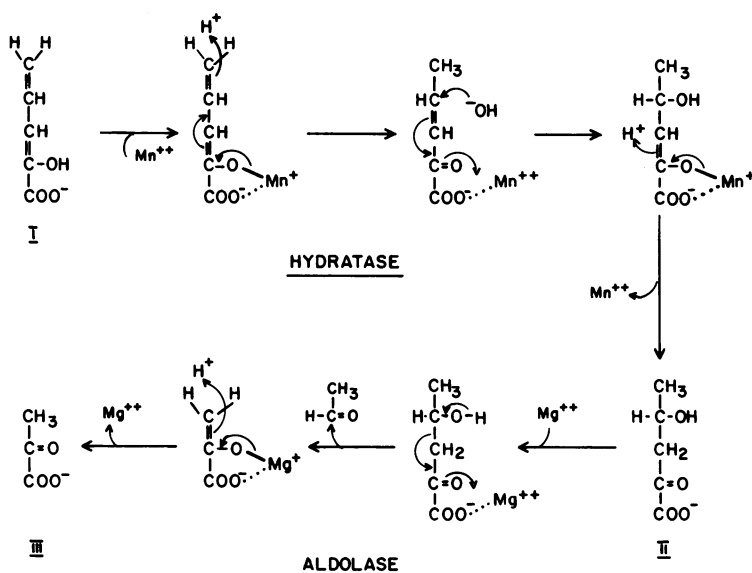
FIG. 8. *Proposed mechanisms for vinylpyruvate hydratase and 4-hydroxy-2-oxopentanoate lyase, presented to show the similarities in the redistribution of electrons when I is hydrated to give II, and when II is cleaved to pyruvate (III) and acetaldehyde.*

action appear to be very similar. In this case, therefore, it is not so improbable that an early enzyme of the sequence could be modified, through mutation in a tandem gene, to permit attack upon the next metabolite as the degradative pathway evolved. Other objections to evolution by tandem gene duplication have stressed that catabolites are often chemically unstable and may never be encountered outside the particular metabolic pathway under consideration (16). These features are well illustrated by the intermediates of *meta*-fission routes: for example, compounds III and IV of Fig. 7 are labile in aqueous solution, they are not metabolized by other living forms as far as is known, and only one enantiomer of each serves as substrate for its aldolase. However, once *meta*-fission of the benzene nucleus has occurred, each one of the compounds of the pathway is metabolized by an enzyme that appears to resemble the others in the sequence with regard to the catalytic mechanism employed. These objections are therefore less impressive for this system than when applied to other catabolic routes that function with dissimilar enzymes.

ACKNOWLEDGMENTS

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