*Biochem. J.* (1971) **122**, 29–40
*Printed in Great Britain*



# The Enzymic Degradation of Alkyl-Substituted Gentisates, Maleates and Malates

By D. J. HOPPER,* P. J. CHAPMAN and S. DAGLEY
*Department of Biochemistry, University of Minnesota, St Paul, Minn.* 55101, *U.S.A.*

(*Received* 13 *November* 1970)

1. Cell-free extracts, prepared from a non-fluorescent *Pseudomonas* grown on *m*-cresol, oxidized gentisate and certain alkyl-substituted gentisates with the consumption of 1 mol of oxygen and the formation of 1 mol of pyruvate from 1 mol of substrate. 2. In addition to pyruvate, malate was formed from gentisate; citramalate was formed from 3-methylgentisate and 4-methylgentisate; 2,3-dimethylmalate was formed from 3,4-dimethylgentisate. 3. One enantiomer, D-(−)-citramalate, was formed enzymically from 3-methylgentisate, 4-methylgentisate and citraconate. L-(+)-Citramalate was formed from mesaconate by the same extracts. When examined as its dimethyl ester by gas–liquid chromatography, enzymically formed 2,3-dimethylmalate showed the same behaviour as one of the two racemates prepared from the synthetic compound. 4. Maleate, citraconate and 2,3-dimethylmaleate were rapidly hydrated by cell extracts, but ethylfumarate and 2,3-dimethylfumarate were not attacked. 5. Cell extracts oxidized 1,4-dihydroxy-2-naphthoate to give pyruvate and phthalate. 6. Alkylgentisates were oxidized by a gentisate oxygenase (EC 1.13.1.4) present in *Pseudomonas* 2,5. The ring-fission products were attacked by maleylpyruvase, but not by fumarylpyruvase, and their u.v.-absorption spectra were those expected for alkyl-substituted maleylpyruvates. 7. When supplemented with ATP, CoA, succinate and $Mg^{2+}$ ions, an enzyme system from cells grown with 2,5-xylenol formed pyruvate from D- but not from L-citramalate. Extracts from cells grown with DL-citramalate or with itaconate attacked both D- and L-citramalate; other alkylmalates were cleaved in similar fashion to give pyruvate or 2-oxobutyrate. 8. These results accord with a general sequence of reactions in which the benzene nucleus of an alkylgentisate is cleaved to give an alkyl-substituted maleylpyruvate. The ring-fission products are hydrolysed to give pyruvate, plus alkylmalic acids which then undergo aldol fissions, probably as their CoA esters. In *Pseudomonas* 2,5 several homologous sequences of this general type appear to be catalysed by a single battery of enzymes with broad substrate specificities, whereas the metabolic capabilities of the fluorescent *Pseudomonas* 3,5 are more restricted. 9. Intact cells of both organisms metabolize D-malic acid by reactions that have not been elucidated, but are different from those which degrade alkylmalates.

Two species of *Pseudomonas*, which grew at the expense of *m*-cresol and other substituted phenols, were shown to oxidize each growth substrate to form a gentisate (Hopper & Chapman, 1971). One of these organisms, *Pseudomonas* 2,5, is known to degrade gentisate (2,5-dihydroxybenzoate) to give pyruvate and D-malate (Hopper, Chapman & Dagley, 1968). In the present investigation we show that extracts of *Pseudomonas* 2,5 convert alkylgentisate into pyruvate and an alkylmalate; moreover, the D isomer of citramalate is formed from either 3-methylgentisate or 4-methylgentisate. A cell-free system that attacked the D, but not the L, isomer of citramalate was studied, and a general scheme is proposed for the metabolism of alkyl-substituted gentisates.

* Present address: Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

## MATERIALS AND METHODS

*Growth of organisms and preparations of cell-free extracts.* The methods used for the maintenance and growth of *Pseudomonas* 2,5 (N.C.I.B. 9867) and *Pseudomonas* 3,5, and for preparing $(NH_4)_2SO_4$-treated extracts, were those described by Hopper & Chapman (1971).

*Partial purification of gentisate oxygenase, maleylpyruvase and fumarylpyruvase.* Gentisate oxygenase (EC

1.13.1.4) was required for preparing solutions of ring-fission products and we are indebted to Dr P. J. Geary for the procedure adopted. Since our sole purpose was to obtain an active preparation free from hydrolases (maleylpyruvase and fumarylpyruvase), specific activities were not determined. A neutralized solution of $(NH_4)_2SO_4$ was added to 21ml of crude extract, prepared from 7g (wet wt.) of *Pseudomonas* 2,5 grown with *m*-cresol, and the protein that precipitated between 40% and 70% saturation with $(NH_4)_2SO_4$ was collected by centrifugation. The precipitate was then dissolved in a mixture of 19ml of 0.1M-phosphate buffer ($KH_2PO_4$, pH7.1) with 1ml of saturated $(NH_4)_2SO_4$. This solution (pH6.8) was applied to a column (5.5cm × 95cm) of Sephadex G-150, which had been pre-equilibrated with buffer+$(NH_4)_2SO_4$, and fractions (16ml) were eluted at 4°C with the same buffer at a flow rate of 39ml/h. Gentisate oxygenase activities were measured by means of an oxygen electrode in the apparatus described by Dagley, Geary & Wood (1968). Since the enzyme was inactive until preincubated with $Fe^{2+}$ ions, 0.9ml of each fraction was incubated at 30°C for 30min with 0.1ml of 0.01M-ferrous ammonium sulphate before assays were performed. The oxygen-electrode chamber contained 6.5ml of 0.1M-phosphate buffer ($KH_2PO_4$, pH7.0), 0.1ml of 0.01M-gentisate and 0.5ml of the activated enzyme. Gentisate oxygenase was found only in fractions 53–65 inclusive. Fractions 54–64 were pooled, the precipitate obtained by adding $(NH_4)_2SO_4$ was dissolved in the mixture of phosphate buffer and $(NH_4)_2SO_4$ used earlier, and the enzyme was used as follows to prepare maleylpyruvate from gentisate. First the substrate was oxidized in 20min, with the uptake of $O_2$ expected for complete ring-fission, in two large Warburg vessels, each of which contained: 10ml of 0.1M-phosphate buffer ($KH_2PO_4$, pH7.0), 0.5ml of $Fe^{2+}$-activated gentisate oxygenase and 1.0ml of 0.1M-gentisate. From one reaction mixture, maleylpyruvic acid was then isolated as its ammonium salt; and from the other, fumarylpyruvic acid was obtained after treatment with $H_2SO_4$. The procedures of Lack (1959) were used in both isolations. These two compounds were used to determine maleylpyruvase and fumarylpyruvase in later fractions from the Sephadex G-150 column: sufficient substrate was added to 3.0ml of 0.1M-phosphate buffer, pH7.0, to give an extinction at 330nm of about 0.4, and the decrease in extinction was observed when a measured volume of enzyme solution was added. Maleylpyruvase was found in fractions 72–93 (maximum activity at fraction 83), fumarylpyruvase in fractions 78–105 (maximum fraction 91). Fraction 99 was chosen for experiments in which fumarylpyruvase free from maleylpyruvase was required. The relative amounts of the two enzymes present in a crude extract depended on the growth substrate: cells grown with *m*-cresol, 3,5-xylenol and 3-ethyl-5-methylphenol contained nearly twice as much fumarylpyruvase as maleylpyruvase, whereas this ratio was depressed to about one-tenth when 2,5-xylenol and 2,3,5-trimethylphenol were used as growth substrates.

*Chromatography.* Dinitrophenylhydrazones were identified and isolated by means of t.l.c. and paper chromatography with solvent G, benzene–tetrahydrofuran (49:1, v/v) (Byrne, 1965) and also solvents *B* and *C* of Chapman & Hopper (1968).

Chromatography on a Celite column (1cm × 30cm), by the procedure of Swim & Utter (1957), was used to purify malate and alkylmalates which were extracted by ether from the reaction mixtures. Fractions (10ml) were collected from the column and titrated with 0.01M-NaOH, with 0.04% Phenol Red as indicator. The acids were further identified by chromatography on paper by the descending method with solvents *H*, butan-1-ol–acetic acid–water (12:3:5, by vol.) (Smith, 1960); *J*, propan-1-ol–eucalyptol–formic acid–water (10:10:4:1, by vol.) (Cheftel, Munier & Macheboeuf, 1951); *K*, propan-1-ol–aq. $NH_3$ (sp.gr. 0.88) (3:2, v/v) (Isherwood & Hanes, 1953). Chromatograms obtained with solvents *H* and *J* were sprayed with 0.1% Bromocresol Green in 96% ethanol made slightly basic with NaOH (Braun & Geenen, 1962); acids were yellow against a green background. With solvent *K*, 0.03% Methyl Red in 0.05M-borate buffer, pH8.0, was used (Kalbe, 1954); acids were red against a lighter background.

Dicarboxylic acids were also identified as their dimethyl esters by g.l.c. (Dagley, Chapman & Gibson, 1965). The three instruments used for this purpose (Table 3) at different times during the course of this investigation were (*a*) Aerograph A-600C, (*b*) F & M model 810 and (*c*) F & M model 5750. We are indebted to Dr Robert Glass for the use of (*b*) and (*c*).

*Chemicals.* 3-Methylgentisic acid, 4-methylgentisic acid, 3-ethylgentisic acid and 3,4-dimethylgentisic acid were prepared as described by Hopper & Chapman (1971). The following compounds, used in chemical syntheses, were obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.: 2-isopropylphenol, 1-hydroxy-2-naphthoic acid, acetoacetate ethyl ester, 2,3-dimethylmaleic anhydride, 2-methylacetoacetic ethyl ester and isobutyrylacetoacetic ethyl ester. 3-Aminonaphthalene-1,5-disulphonic acid was obtained as the sodium salt from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

3-Isopropylgentisic acid (m.p. 156–157°C) was prepared by oxidizing 2-hydroxy-3-isopropylbenzoic acid with persulphate, by using essentially the same procedure as that of Updegraff & Cassidy (1949). 2-Hydroxy-3-isopropylbenzoic acid was obtained by carboxylating 2-isopropylphenol by the method of Jones (1958); the product gave white crystals from aq. ethanol, m.p. 68–69°C. Croxall, Sowa & Nieuwland (1937) gave m.p. 70–71°C.

6-Methylgentisic acid was obtained by oxidizing 2-methyl-3-hydroxybenzoic acid with persulphate. Sublimation under reduced pressure gave pale-yellow crystals, m.p. 214–216°C (decomp.). Inouye (1954) gave m.p. 214–215°C. 2-Methyl-3-hydroxybenzoic acid was prepared by heating the sodium salt of 3-aminonaphthalene-1,5-disulphonic acid in 50% (w/v) NaOH in a nickel bomb at 270°C (Fieser & Lothrop, 1936). We thank the 3M Company, St Paul, Minn., U.S.A., for making their high-pressure facilities available, and also Mr Milton Sandberg, who conducted the alkaline fusion.

Citramalic acid was prepared by hydrolysis of the cyanohydrin formed from HCN and acetoacetic ethyl ester, by following the procedure of Barker (1962). The product gave white crystals, m.p. 114–115°C, from ethyl acetate–light petroleum (b.p. 60–80°C). Barker (1962) gave m.p. 116.4–117.2°C.

2,3-Dimethylfumaric acid, and the monosodium salt of 2,3-dimethylmaleic acid, were prepared from 2,3-

dimethylmaleic anhydride as described by Couper, Kibler & Lutz (1941).

1,4-Dihydroxy-2-naphthoic acid was obtained by oxidizing 1-hydroxy-2-naphthoic acid with persulphate. Pale-yellow crystals were given from aq. ethanol, m.p. 195–196°C (decomp.). Desai & Sethna (1951) gave m.p. 201–202°C; Homeyer & Wallingford (1942) gave m.p. 200°C (decomp.).

The preparation of 2,3-dimethylmalic acid was similar to that of citramalic acid, 2-methylacetoacetic ethyl ester being used instead of acetoacetic ethyl ester. Examination by g.l.c. of the starting material used for the synthesis showed that it contained 70% of 2-methylacetoacetic ethyl ester and also two other components, one of which, acetoacetic ethyl ester, gave rise to some citramalic acid during the preparation. The synthetic 2,3-dimethylmalic acid was a semi-crystalline syrup, which gave two main peaks and a minor peak (citramalate) when the dimethyl esters were subjected to g.l.c. When the synthetic product was degraded with $H_2SO_4$, under the conditions used by Strassman & Ceci (1963) to establish the structure of α-isopropylmalate, the only DNP-hydrazones obtained were those of ethyl methyl ketone (from 2,3-dimethylmalic acid) and acetone (from citramalate). It was therefore assumed that the two main peaks observed on g.l.c. were the dimethyl esters of *erythro*- and *threo*-2,3-dimethylmalic acid. Calvo, Kalyanpur & Stevens (1962) described the separation of *erythro*- and *threo*-α-isopropylmalic acid on a silicic acid column. Their procedure was adopted for our preparation of 2,3-dimethylmalic acid, on a column 60cm×3cm. However, we observed one major peak, with no separation of the racemate pairs, and one minor peak due to citramalic acid. Accordingly, the fractions constituting the main peak were pooled, extracted with NaOH, acidified and dried. The product was extracted with hot ethyl acetate and evaporated to give a syrup, which partially crystallized when left overnight in a vacuum desiccator. Some separation of the two racemic mixtures was achieved by washing the crystals with ethyl acetate: the syrup was more soluble than the crystals, and these could be removed by filtration. Evaporation of the ethyl acetate gave the syrup again, from which more crystals were obtained. Three crops of crystals were collected, and when the fractions were examined as their dimethyl esters by g.l.c. using instrument (c) (see Table 3) the crystals (referred to later as 'racemate crystals') gave a major peak with retention time of 6.7min and a minor peak at 5.4min. The syrup ('racemate oil') gave a major peak at 5.4min and a minor peak at 6.7min. The crystals were again crystallized from ethyl acetate–light petroleum (b.p. 60–80°C) and when examined by g.l.c. they were found to contain at least 90% of the isomer with a retention time 6.7min. The racemate oil contained about 70% of the isomer with retention time 5.4min, and was later found to be the biologically active form: the racemate crystals were inactive towards cell extracts, The n.m.r. spectra of the racemates differed in detail, but each was consistent with the chemical structure of 2,3-dimethylmalic acid.

2-Ethylmalic acid and 2-ethylfumaric acid were the kind gifts of Dr S. J. Ajl. 2-Isopropylmalic acid was prepared by a procedure similar to that for citramalic acid, with isobutyrylacetoacetic ethyl ester. The product, m.p. 145°C, softened at 141°C. Jungwirth, Gross, Margolin & Umbarger (1963) gave m.p. 146–147°C. The i.r.-absorption spectrum in Nujol was identical with that given by Sai (1968). Degradation with $H_2SO_4$ gave methyl isopropyl ketone, identified as its DNP-hydrazone by t.l.c.

*Analyses.* Pyruvate was determined spectrophotometrically by means of lactate dehydrogenase (EC 1.1.1.27) from Calbiochem, Los Angeles, Calif., U.S.A., and also by the method of Friedemann & Haugen (1943). The optical rotations of compounds were measured in an automatic polarimeter from Bendix Corp., Cincinnati, Ohio, U.S.A.

## RESULTS

*Formation of pyruvate from gentisate and alkyl-substituted gentisates.* The following compounds were previously identified as products of oxidation of substituted phenols by *Pseudomonas* 2,5: gentisate, 3-methylgentisate, 4-methylgentisate, 3-ethylgentisate and 3,4-dimethylgentisate (Hopper & Chapman, 1971). All of these compounds, and also 3-isopropylgentisate, were oxidized by ammonium sulphate-treated extracts of *Pseudomonas* 2,5 grown with *m*-cresol; 1mol of oxygen was consumed for 1mol of each substrate oxidized, and 1mol of pyruvate was formed (Table 1). In these experiments the final volume of each reaction mixture after removal of protein was 10ml, of which 0.5ml was used for assaying pyruvate. To the remaining 9.5ml were added 5ml of 0.1% DNP-hydrazine in 2M-hydrochloric acid, and after incubating at 30°C for 30min, a DNP-hydrazone was extracted and submitted to t.l.c. in solvent *B* and to paper chromatography, by the descending method, in solvent *C*. The only spots shown by each reaction mixture were those with the same $R_F$ values as those of authentic pyruvate DNP-hydrazone.

*Formation of malate and alkylmalates from gentisates.* Five 50ml Erlenmeyer flasks each contained 7ml of ammonium sulphate-treated extract (18mg of protein/ml), 8ml of 0.5M-phosphate buffer, pH7.0, and 1.0ml of 2M-ferrous sulphate. They were shaken for 10min at 30°C and additions were then made of 1.4ml of solutions (0.1M, pH7.0) of the following sodium salts, one substrate being added to each flask: gentisate, 3-methylgentisate, 4-methylgentisate, 3-ethylgentisate and 3,4-dimethylgentisate. When oxidation ceased, as indicated for similar reaction mixtures in Warburg flasks, a second addition of 1.4ml was made, and finally a third addition. Shaking was continued for 30min after oxidation of the last addition of each substrate was complete. Protein was precipitated by adding 20ml of 30% (w/v) metaphosphoric acid and was then removed by centrifuging, 10ml of 2M-hydrochloric acid was also added and each solution was continuously extracted with other for 48h. Ethereal solutions were dried over sodium

sulphate and then evaporated to yield small quantities of greasy solids. When these products were purified by chromatography on Celite (Fig. 1), the overall yield of each malic acid as determined from titres was about 70%. Each was extracted from eluted fractions by shaking with sodium hydroxide, and after acidification, the aqueous solutions were extracted continuously with ether for 48h. The ether was dried with sodium sulphate and then removed to leave small amounts of viscous syrups. Paper chromatography by the descending method in three solvent systems (Table 2) was used to identify these products tentatively as follows: malate, produced from gentisate; citramalate from 3-methylgentisate and 4-methylgentisate; 2-ethylmalate from 3-ethylgentisate; 2,3-dimethylmalate from 3,4-dimethylgentisate. These results were confirmed by g.l.c. of the dimethyl esters (Table 3) and were also indicated by the elution profiles for the acids when chromatographed on Celite (Fig. 1). The profile for the product from 2,3-dimethylgentisate, which has been omitted from Fig. 1, was almost coincident with that for 2-ethylmalate. Synthetic 2,3-dimethylmalic acid consists of two racemic mixtures, which can be separated as an oil and a crystalline racemate as described in the Materials and Methods section. The oil, but not the crystals, behaved the same as enzymically formed 2,3-dimethylmalic acid when the ethyl esters were examined by g.l.c.

*Enzyme-catalysed hydration of unsaturated dicarboxylic acids.* Nucleic acids were removed from a crude extract of *m*-cresol-grown cells by adding 0.1 vol. of 2% (w/v) protamine sulphate at 0°C; the precipitate obtained by standing for 30min was then centrifuged and discarded. This procedure, when repeated twice, gave an extract that could be used to follow enzyme-catalysed disappearances of various unsaturated acids by measuring accompanying decreases in extinctions at 250nm. The *cis*-unsaturated acids, maleic acid, citraconic acid and 2,3-dimethylmaleic acid, rapidly disappeared but the *trans* acids, ethylfumaric acid and 2,3-dimethylfumaric acid, were not metabolized. However, fumaric acid and mesaconic acid were rapidly attacked. These results were confirmed for reaction mixtures, each of which contained, in 3ml, an ammonium sulphate-treated extract (70mg of protein) and 10mg of one of the unsaturated acids. One set of reaction mixtures was incubated at 30°C for 2.5h and then boiled, and a control set was boiled immediately after the extract was added. Precipitated protein was removed by centrifugation, solutions were acidified with sulphuric acid and 7g of ether-washed Celite was added to each solution. The Celite was extracted twice by washing with

Table 1. *Formation of pyruvate by enzymic oxidation of gentisic acids*

The main compartments of Warburg flasks contained, in 3.0ml, 60$\mu$mol of phosphate buffer, pH7.0, 18$\mu$mol of ferrous sulphate and 18.4mg of cell-extract protein. The centre wells contained 0.2ml of 20% (w/v) KOH, and reactions were started by tipping 5$\mu$mol of substrate from the side arms. The gas phase was air and the temperature was 30°C. When uptake of $O_2$ ceased, protein was removed from reaction mixtures and the amount of pyruvate formed was determined by the method of Friedemann & Haugen (1943).

| Substrate (5$\mu$mol) | Uptake of $O_2$ ($\mu$mol) | Pyruvate formed ($\mu$mol) |
|---|---|---|
| Gentisate | 5.0 | 5.2 |
| 3-Methylgentisate | 5.0 | 5.0 |
| 4-Methylgentisate | 4.8 | 4.9 |
| 3-Ethylgentisate | 5.0 | 5.0 |
| 3,4-Dimethylgentisate | 4.7 | 4.7 |
| 3-Isopropylgentisate | 4.8 | 4.9 |

Table 2. *Chromatographic identification of malates formed enzymically from gentisates*

Details of solvents *H*, *J* and *K*, and reagents used for spraying, are given in the Materials and Methods section.

| | $R_F$ | | |
|---|---|---|---|
| Solvent ... | *H* | *J* | *K* |
| Compound | | | |
| Malate | 0.55 | 0.33 | 0.23 |
| Product from gentisate | 0.54 | 0.33 | 0.23 |
| Citramalate | 0.65 | 0.47 | 0.31 |
| Product from 3-methylgentisate | 0.66 | 0.46 | 0.30 |
| Product from 4-methylgentisate | 0.65 | 0.47 | 0.31 |
| 2-Ethylmalate | 0.71 | 0.62 | 0.36 |
| Product from 3-ethylgentisate | 0.70 | 0.62 | 0.36 |
| 2,3-Dimethylmalate* | 0.71 | 0.65 | 0.34 |
| Product from 3,4-dimethylgentisate | 0.67 | 0.61 | 0.35 |

* Both racemates were present in the sample.

Table 3. *Gas–liquid chromatography of the dimethyl esters of malic acids from gentisic acids*

The three instruments used, (a) (b) and (c), are described in the Materials and Methods section. The temperature of each column was 150°C. Stationary phases were: (a) 5% butane-2,3-diol succinate; (b) and (c), diethyleneglycol succinate. — denotes that no measurements were made. Values in parentheses are for minor peaks.

| Dimethyl ester of: | Retention time (min) (a) | (b) | (c) |
|---|---|---|---|
| Malic acid | 13.5 | — | — |
| Product from gentisic acid | 13.5 | — | — |
| Citramalic acid | 4.9 | — | 5.8 |
| Product from 3-methylgentisic acid | 4.9 | — | — |
| Product from 4-methylgentisic acid | 4.9 | — | — |
| 2-Ethylmaleic acid | — | 2.9 | — |
| Product from 3-ethylgentisic acid | — | 2.9 | — |
| 2,3-Dimethylmalic acid (racemate oil) | — | — | 5.4 (6.7) |
| 2,3-Dimethylmalic acid (racemate crystals) | — | — | 6.7 (5.4) |
| Product from 3,4-dimethylgentisic acid | — | — | 5.4 |

200ml of ether (Swim & Utter, 1957), and the ether extract, when dried over sodium sulphate, was evaporated to leave residues, which were then chromatographed in solvents *H* and *J*, as described for Table 2. The control set of mixtures gave only the initial substrates, except that 2,3-dimethylmaleate was not detected, probably because it formed an anhydride on acidification (Couper *et al.* 1941). Chromatograms of the reaction products showed that fumarate and maleate gave malate; mesaconate and citraconate gave citramalate; and dimethylmaleate gave 2,3-dimethylmalate. In the reaction mixtures containing fumarate and mesaconate, a proportion of each substrate was recovered unchanged. No detectable amounts of malic acids were given by the *trans*-unsaturated acids, ethylfumaric acid and dimethylfumaric acid, which also appeared unchanged on the chromatograms.

*Configurations of enantiomers of citramalate formed from 3-methylgentisate, 4-methylgentisate, citraconate and mesaconate.* Hopper *et al.* (1968) showed that cell-free extracts of *Pseudomonas* 2,5 converted gentisate and maleate into pyruvate and D-malate, whereas L-malate was formed from fumarate. Since 3-methylgentisate, 4-methylgentisate, citraconate and mesaconate were all found to give citramalate, reactions were run on a larger scale in order to provide sufficient citramalate for its configuration to be determined in each case. The procedure was similar to that adopted when the compounds of Fig. 1 were isolated, except that five successive additions of 10ml of 0.1M-substrate were made at intervals of 30min, a single substrate being added to each flask (250ml) containing 50ml of an ammonium sulphate-treated extract, 10ml of 0.5M-phosphate buffer, pH7.0, and 2ml of 0.2M-ferrous sulphate. The previous isolation procedure was followed, consisting of ether extraction and Celite chromatography on a column 40cm × 1.7cm. The reaction mixtures from 3-methylgentisate,

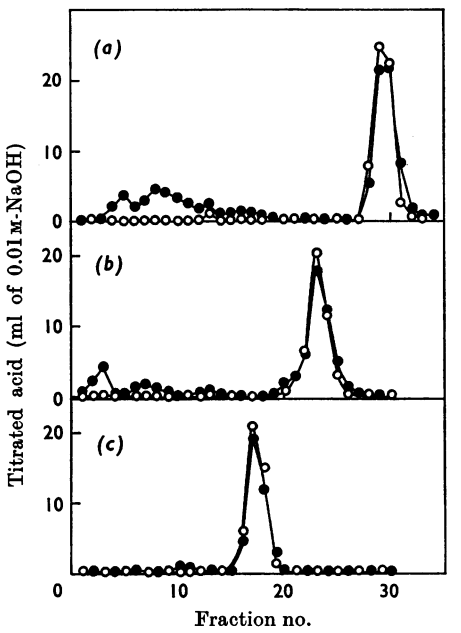



Fig. 1. Chromatography on Celite. Carboxylic acids extracted from reaction mixtures were applied to the column, eluted and titrated in fractions (10ml). (*a*) Product from gentisate (●), authentic malate (○); (*b*) product from either 3- or 4-methylgentisate (●), authentic citramalate (○); (*c*) product from 3-ethylgentisate (●), authentic 2-ethylmalate (○). Elution peaks were observed at the following percentages (v/v) of butan-1-ol in chloroform: 50% (*a*); 25% (*b*); 15% (*c*).

Table 4. *Optical rotations of samples of citramalate prepared enzymically from mesaconate, citraconate, 3-methylgentisate and 4-methylgentisate*

Samples of citramalate were isolated and crystallized as described in the text. Rotations of 3% (w/v) solutions were measured in the presence and absence of the citrate–molybdate mixture of Krebs & Eggleston (1943) at 24°C in a polarimeter with a tube length of 2cm.

| Citramalate from: | $[\alpha]_D$ (°) Molybdate absent | $[\alpha]_D$ (°) Molybdate present |
|---|---|---|
| Mesaconate | +21 | +1282 |
| Citraconate | −21 | −1304 |
| 3-Methylgentisate | −20.6 | −1279 |
| 4-Methylgentisate | −20.6 | −1274 |

4-methylgentisate and citraconate all gave one major elution peak, coincident with that of authentic citramalate. From mesaconate, two peaks were given, corresponding to mesaconate and citramalate. Four samples of citramalate were obtained from these fractions, and each was crystallized as described by Barker & Blair (1962). (−)-Citramalate was obtained from citraconate, 3-methylgentisate and 4-methylgentisate, whereas mesaconate gave (+)-citramalate (Table 4). For 4.4% (w/v) (+)-citramalate, Barker & Blair (1962) gave $[\alpha]_D$ 23.2°, enhanced in citrate–ammonium molybdate solutions to 1230°.

The identities of the isolated materials were confirmed by chemical degradation and by examination of i.r.-absorption spectra. The spectra of the samples in Nujol were identical, but they differed from that of authentic DL-citramalate. However, the solid-state i.r.-absorption spectra of racemic amino acids may differ markedly from those of the enantiomers (Koegel, McCallum, Greenstein, Winitz & Birnbaum, 1957; Shaw, Borkenhagen & Talalay, 1965). Accordingly (+)-citramalate from mesaconate was crystallized with equal quantities of the three samples of (−)-citramalate isolated from the other sources, and spectra were then identical with that of authentic DL-citramalate. The procedure adopted for identification by chemical degradation was similar to that used for α-isopropylmalate by Strassman & Ceci (1963): treatment of citramalate with cold sulphuric acid liberates carbon monoxide and forms acetoacetic acid, which on warming decarboxylates to give acetone. To 30mg portions of each product and authentic citramalic acid was added 0.3ml of conc. sulphuric acid. After standing for 1h at room temperature, 3.0ml of water was added to each mixture, contained in small flasks with side arms that dipped into 50ml of 0.1% DNP-hydrazine. Flasks were heated on a steam bath for 20min, their contents were added to the DNP-hydrazine solutions, and each DNP-hydrazone formed was extracted with ethyl acetate. The solids obtained by evaporating the solvent were applied to preparative thin-layer plates, and when chromatographed in solvent A gave main bands which were then eluted with ethyl acetate. Each DNP-hydrazone gave a single spot with the same $R_F$ (0.38) as acetone DNP-hydrazone when examined by t.l.c. in the same solvent.

*Oxidation of 1,4-dihydroxy-2-naphthoate by cell extracts.* The foregoing results for the metabolism of 3-methylgentisate and 4-methylgentisate support Scheme 1 in which ring-fission products are not isomerized, and *cis* forms are shown to be hydrolysed and hydrated. Thus, although the *trans*-unsaturated dicarboxylic acid, mesaconate, is hydrated by extracts, this reaction does not appear to take part in the sequence since the product of hydration, L-citramalate, is not formed from the gentisates. It was of interest to find that 1,4-dihydroxy-2-naphthoate was metabolized to pyruvate by extracts, since the chemical structure of the substrate precludes isomerization (Scheme 1). Pyruvate formed enzymically was isolated as its DNP-hydrazone and identified by t.l.c. and paper chromatography as described above. When the reaction took place in a respirometer, 5μmol of substrate gave 3.55μmol of pyruvate, and 3.75 μmol of oxygen was consumed. An uptake of oxygen lower than the theoretical was probably due to the presence of the quinone in the substrate, and also to its rapid non-enzymic formation in the incubation mixture. The second oxidation product was isolated by making five successive additions of 5.0ml of 0.5% 1,4-dihydroxy-2-naphthoic acid in 0.5M-phosphate buffer, pH7.0, to a 125ml Erlenmeyer flask, which was shaken at 30°C and contained the following: 10ml of 0.5M-phosphate buffer, pH7.0, 20ml of an ammonium sulphate-treated extract of *Pseudomonas* 2,5 grown with *m*-cresol, and 1.0ml of 0.18M-ferrous sulphate. The additions were made at intervals of 15min, the flask was shaken for a further 2h and protein was then precipitated by adding 30ml of 30% (w/v) metaphosphoric acid and removed by centrifuging. The solution was further acidified with sulphuric acid, extracted continuously with ether for 12h and the ethereal solution dried over sodium sulphate. Evaporation of the ether gave about 100mg of a yellow solid which, on crystallizing from water after treatment with charcoal, gave white crystals, m.p. 217–218°C (heated at 6°C/min). Hickinbottom & Garwood (1956) observed that the m.p. of phthalic acid (about 200°C) varies very much with rate of heating. Heilbron & Bunbury (1953) gave m.p. 231°C for rapid heating. Our isolated com-

pound gave m.p. 222–224°C when mixed with authentic phthalic acid, m.p. 222–224°C. The isolated compound had the same i.r.-absorption spectrum as phthalic acid; both materials gave a single spot of $R_F$ 0.36, with some tailing, when run in thin-layer chromatograms with benzene–dioxan–acetic acid (90:25:4, by vol.) as solvent (Pastuska, 1961).

*Formation and degradation of products of ring-fission of gentisates.* An ammonium sulphate-treated extract of *Pseudomonas* 2,5 grown with *m*-cresol was passed through Sephadex G-150 and separate fractions were collected, which contained gentisate oxygenase, fumarylpyruvase and maleylpyruvase as described in the Materials and Methods section. Activated gentisate oxygenase (0.02ml) was added to reaction mixtures that contained 0.1$\mu$mol of one of the following substrates in 3ml of 0.1M-phosphate buffer, pH7.0: gentisate, 3-methylgentisate, 3-ethylgentisate and 3-isopropylgentisate. The light-absorption at 340nm increased until each substrate was consumed and then remained at a stationary value, indicating that hydrolases were not present in the gentisate oxygenase preparation. The spectra of all the ring-fission products were similar, having $\lambda_{max.}$ at 330–340nm in alkali; the u.v. light-absorption was largely abolished on acidification, as expected for compounds related structurally to maleylpyruvate (Lack, 1959), i.e. having *cis* configurations. The spectrum of the ring-fission product from 4-methylgentisate had $\lambda_{max.}$ 320nm in alkali, with light-absorption abolished on acidification. Additions of fraction 99 from the Sephadex G-150 column, which contained only fumarylpyruvase, had no effect on any of the ring-fission products. However, maleylpyruvase from fraction 82 rapidly degraded the products from 3-methylgentisate, 4-methylgentisate and 3-ethylgentisate in addition to hydrolysing maleylpyruvate formed from gentisate.

Ammonium sulphate-treated extracts of *Pseudomonas* 2,5 and *Pseudomonas* 3,5 were activated by incubating with mM-ferrous ammonium sulphate for 1h and rates of oxidation of 0.15mM-gentisates were measured (Table 5). Neither extract oxidized 6-methylgentisate. For the other five gentisates, rates of oxidation by the *Pseudomonas* 2,5 extract varied less than twofold, but by contrast the extract of *Pseudomonas* 3,5 oxidized gentisate about seven times faster than 3,4-dimethylgentisate and 70 times faster than 4-methylgentisate.

*Enzymic degradation of alkyl-substituted malic acids.* Cooper & Kornberg (1964) showed that extracts of another species of *Pseudomonas* converted DL-citramalate into pyruvate and acetyl-CoA in the presence of $Mg^{2+}$ ions, succinate, CoA and ATP. We found that extracts of *Pseudomonas* 2,5 grown with 2,5-xylenol readily cleaved D-citramalate to pyruvate, which was assayed spectrophotometrically with lactate dehydrogenase. No significant reaction was observed when ATP, CoA or succinate were omitted singly, and the complete reaction system did not attack L-citramalate (Fig. 2*a*). However, when the cells were grown with DL-citramalate, extracts then attacked each of the separate enantiomers (Fig. 2*b*) and DL-citramalate was also readily metabolized. Further, extracts of itaconate-grown cells attacked both enantiomers when all the above-mentioned cofactors were present. The racemate oil of 2,3-dimethylmalate, which was identical with enzymically formed 2,3-dimethylmalate (Table 3), was also rapidly metabolized to pyruvate by the complete enzyme system, whereas a slow reaction of only 4% of the added crystalline racemate was observed, a result that may be attributed to contamination by the other racemate. 2-Ethylmalate was shown in later experiments to yield 2-oxobutyrate instead of pyruvate. However, it was possible to demonstrate the cofactor requirements of this reaction by using a modified assay in which the addition of lactate dehydrogenase was increased to 20$\mu$l; this enzyme attacks 2-oxobutyrate, although the rate is slower than that for pyruvate. When either CoA or ATP was omitted, rates of oxidation of NADH were decreased to less than one-tenth, and in the absence of succinate the rate was less than one-half of that for the complete system.

Oxo acids were further identified by incubating the cell extract, supplemented with cofactors as in Fig. 2, with various malic acids present as substrates,

Table 5. *Oxidation of gentisates by extracts of* Pseudomonas 2,5 *and* Pseudomonas 3,5

Ammonium sulphate-treated extracts were prepared from *Pseudomonas* 2,5 grown with *m*-cresol (16.6mg of extract protein/ml) and *Pseudomonas* 3,5 (19.8mg of extract protein/ml). Extracts were activated with ferrous ammonium sulphate and rates of oxidation of gentisates were measured with an oxygen electrode. The electrode chamber contained 6.5ml of 0.05M-phosphate buffer, pH 7.0, 0.1ml of substrate (0.01M) and 0.1ml of cell extract.

| Substrate oxidized | Rate of oxidation ($\mu$l of $O_2$/min) | |
|---|---|---|
| | Extract of *Pseudomonas* 2,5 | Extract of *Pseudomonas* 3,5 |
| Gentisate | 1.55 | 0.99 |
| 3-Methylgentisate | 2.78 | 1.02 |
| 4-Methylgentisate | 1.90 | 0.014 |
| 3-Ethylgentisate | 2.36 | 0.62 |
| 3,4-Dimethylgentisate | 1.55 | 0.13 |
| 6-Methylgentisate | 0.00 | 0.00 |

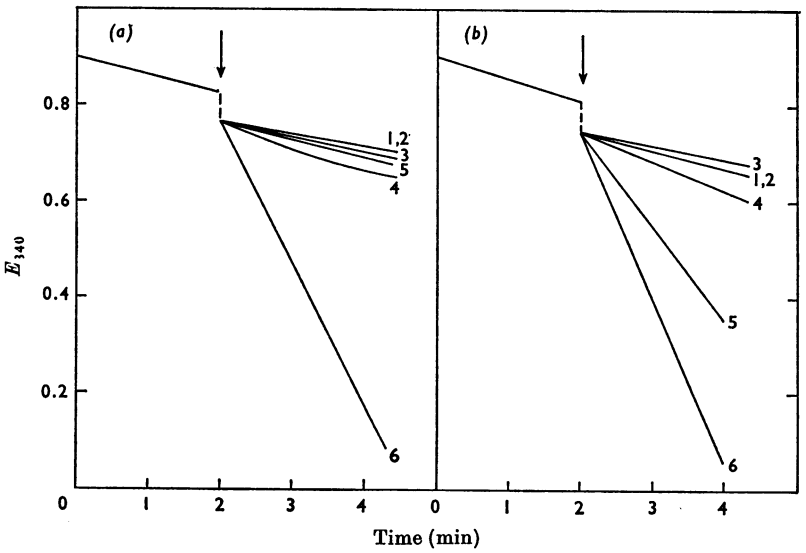
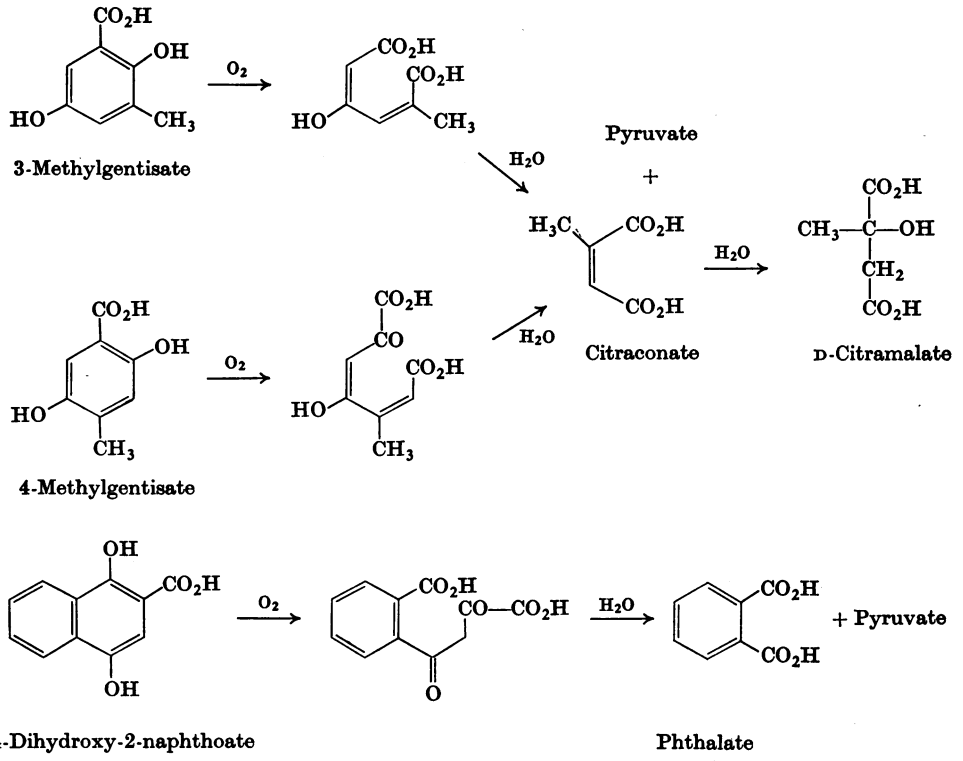



Fig. 2. Formation of pyruvate from citramalate by extracts of *Pseudomonas* 2,5 grown with 2,5-xylenol (*a*) and DL-citramalate (*b*). Cuvettes (2ml) contained 85$\mu$mol of tris–HCl buffer, pH8.0, 1.5$\mu$mol of CoA, 1mg of NADH, 1$\mu$l of lactate dehydrogenase and 1mg of cell-extract protein. Cells were broken in a Hughes press and extracts centrifuged at 100000*g* for 90min. Citramalate (5$\mu$mol) was added at points indicated by arrows, except where stated. The following were omitted singly from the complete system: 1, citramalate; 2, ATP; 3, CoA; 4, succinate. In (*a*) D-citramalate was present as substrate for reactions 2, 3 and 4; in (*b*) DL-citramalate was present. Decreases in $E_{340}$ for complete systems were obtained by adding the following substrates: 5, L-citramalate; 6, D-citramalate.

for 1h. Protein was then precipitated by adding 1ml of 30% (w/v) metaphosphoric acid and removed by centrifuging. Solutions were incubated at 30°C for 30min with 5ml of 0.1% DNP-hydrazine in 2M-hydrochloric acid, and the resulting DNP-hydrazones were extracted into ethyl acetate, then into 10% (w/v) sodium carbonate and finally, after acidification, into ethyl acetate. The solutions were dried over sodium sulphate and evaporated, and the residues were examined by t.l.c. in solvent *B*. No major spots were observed when no substrate was added to the reaction mixture, or when D-malate, the racemate crystals of 2,3-dimethylmalate or 2-isopropylmalate were added as substrates. Large spots corresponding to authentic pyruvate DNP-hydrazone were given from reaction mixtures containing D-citramalate, L-citramalate and the racemate oil of 2,3-dimethylmalate. A large spot corresponding to authentic 2-oxobutyrate DNP-hydrazone was given from 2-ethylmalate. Since no glyoxylate DNP-hydrazone was formed from D-malate, even when 5$\mu$mol of semicarbazide was also added to the reaction mixture, it appears that D-malate is not attacked by the enzyme system that degrades alkylmalates. The major spots were scraped from the plates, eluted with ethyl acetate, and the identities of the DNP-hydrazones were confirmed by paper chromatography by the descending method with solvent *C*.

## DISCUSSION

Hopper & Chapman (1971) studied the oxidation of various substituted phenols by a non-fluorescent organism, *Pseudomonas* 2,5. On addition of $\alpha\alpha'$-bipyridyl to inhibit their further degradation, alkyl-substituted gentisates were found to accumulate from alkylphenols when metabolized by intact cells, or by cell extracts supplemented with NADH. In the present work we showed that cell extracts converted 1mol of each gentisic acid into 1mol of pyruvate with the consumption of 1mol of oxygen. The second reaction product was malate, or an alkylmalate, as required by a reaction sequence in which hydrolysis and hydration follow ring-fission of the gentisate. Thus, as shown in Scheme 1, 3-methylgentisate and 4-methylgentisate would give rise to citramalate, as was found; likewise 3-ethylgentisate gave 2-ethylmalate. Extracts of *Pseudomonas* 2,5 converted gentisate into D-malate (Hopper *et al.* 1968) and the experiments of Table 4 showed that both 3-methylgentisate and 4-methylgentisate gave (−)-citramalate, which is known to exist in the D (or *R*) configuration (von




Scheme 1.

der Mühll, Settimj, Weber & Arigoni, 1965). In Scheme 1, the ring-fission product does not undergo *cis–trans* isomerization, such as Lack (1959) found when gentisate was metabolized by another species of *Pseudomonas*; instead, citraconate (2-methylmaleate) is hydrated.

The following evidence supports the view that Scheme 1 is a particular application (to methylgentisates) of a general metabolic pathway that *Pseudomonas* 2,5 employs for the degradation of a series of homologous substrates. (1) Extracts that metabolized the methylgentisates to a single isomer, D(−)-citramalate (Table 4), contained enzymes that hydrated mesaconate (2-methylfumarate) and citraconate to give L-citramalate and D-citramalate respectively. Hopper *et al.* (1968) likewise found that extracts of this organism formed L-malate from fumarate, whereas D-malate was given exclusively from gentisate and maleate. (2) Of the *trans*-unsaturated dicarboxylic acids, only fumarate and mesaconate were metabolized; extracts contained fumarase; since Katsuki *et al.* (1962) found that *Pseudomonas fluorescens* metabolized fumarate and mesaconate by separate enzymes, our extracts may also contain a constitutive mesaconase. Ethylfumarate and 2,3-dimethylfumarate were not attacked by extracts whereas 2,3-dimethylmaleate was readily converted into 2,3-dimethylmalate. (3) The ring-fission products of 3-methylgentisate, 4-methylgentisate and 3-ethylgentisate closely resembled maleylpyruvate, and differed from fumarylpyruvate, in their spectral properties. Maleylpyruvase, free from fumarylpyruvase, attacked each product, but fumarylpyruvase attacked none. Further, extracts catalysed the oxidation of 1,4-dihydroxy-2-naphthoate to give a ring-fission product, which, although incapable of undergoing *cis–trans* isomerization, was readily hydrolysed to give pyruvate and phthalate (Scheme 1). This reaction, which could enable $^{14}C$ to be located at various positions in 1,4-dihydroxy-2-[$^{14}C$]naphthoic acid by enzymic degradation, may prove useful for studies of the biosynthesis of bacterial menaquinones since this naphthalene compound has been suggested as a biosynthetic intermediate (Robins, Campbell & Bentley, 1970; Dansette & Azerad, 1970).

Stereospecificity was shown by the enzyme that

degraded, as well as by those that formed, D-citramalate. Extracts of cells grown with 2,5-xylenol rapidly attacked the D, but not the L, isomer. The enzymic synthesis and degradation of 2,3-dimethylmalate was also stereospecific, insofar as an isomer in the racemate oil was formed by extracts (Table 3) and was also degraded by them when cofactors were provided. We did not identify the active isomer of 2,3-dimethylmalate, but isomers in the crystalline racemate were not biologically active. The ability to metabolize L-citramalate was acquired by growing the cells with itaconate or DL-citramalate. Since ATP, CoA and succinate were all needed for activity, and pyruvate was identified as one product, it is assumed that the enzymic degradation of either isomer of citramalate is represented thus:

$$\text{Citramalate} + \text{succinyl-CoA} \rightarrow \text{pyruvate} + \text{acetyl-CoA} + \text{succinate} \quad (1)$$

However, the direct participation of succinyl-CoA was not proved, and attempts to trap acetyl-CoA as citrate were hindered by the presence in cell extracts of a powerful oxaloacetate decarboxylase. Cooper & Kornberg (1964) showed that extracts of a *Pseudomonas* grown with itaconate formed pyruvate and acetyl-CoA from DL-citramalate; the cofactors required were the same as those in the present investigation. Pyruvate was isolated as its DNP-hydrazone from D-citramalate, L-citramalate and the racemate oil of 2,3-dimethylmalate when these compounds were metabolized by supplemented extracts of *Pseudomonas* 2,5 grown with DL-citramalate; 2-oxobutyrate was given from 2-ethylmalate. The following equations, similar to (1), are therefore proposed:

$$\text{2-Ethylmalate} + \text{succinyl-CoA} \rightarrow \text{2-oxobutyrate} + \text{acetyl-CoA} + \text{succinate} \quad (2)$$

$$\text{2,3-Dimethylmalate} + \text{succinyl-CoA} \rightarrow \text{pyruvate} + \text{propionyl-CoA} + \text{succinate} \quad (3)$$

The formation of propionyl-CoA was not directly demonstrated; but propionate was readily oxidized by cells grown with 2,3,5-trimethylphenol, which gives rise to 3,4-dimethylgentisate (Hopper & Chapman, 1971) and hence to 2,3-dimethylmalate by reactions analogous to those of Scheme 1. By contrast, cells grown with 3,5-xylenol did not oxidize propionate. The growth substrate in this case is oxidized, first to 3-methylgentisate and then to citramalate, which would furnish acetyl-CoA, and not propionyl-CoA.

It may be noted that an alkylmalate, namely 2-isopropylmalate, is an intermediate in the biosynthesis of leucine and is formed when acetyl-CoA condenses with 2-oxoisovalerate (Strassman & Ceci, 1963). Rabin, Salamon, Bleiweis, Carlin & Ajl (1968) have presented evidence that enzymes of the leucine-biosynthetic pathway catalyse the formation of 2-ethylmalate from acetyl-CoA and 2-oxobutyrate, and also certain reactions of 3-ethylmalate in *Pseudomonas aeruginosa*. It is most improbable that reaction (2) in *Pseudomonas* 2,5 is catalysed, in reverse, by an enzyme that operates for leucine biosynthesis, since 2-isopropylmalate was not a substrate for the complete system that catalyses reaction (2). Moreover, Rabin *et al.* (1968) have concluded that the cleavage of 2-ethylmalyl-CoA to give acetyl-CoA and 2-oxobutyrate, which has been observed for the organism they studied, may be catalysed by the citramalyl-CoA cleavage enzyme of Cooper & Kornberg (1964). This enzyme, as we have indicated, appears to be present in *Pseudomonas* 2,5 when grown with itaconate or DL-citramalate.

D-Malate is formed endogenously from gentisate but is not metabolized by reactions analogous to those investigated for alkylmalates. The following enzymes concerned with the metabolism of malic acid were shown to be present in cell-free extracts of our organisms: a soluble L-malate–NAD oxidoreductase (EC 1.1.1.37); a 'particle-bound' malate dehydrogenase that did not require $NAD^+$; and L-malate–NADP oxidoreductase (decarboxylating) (EC 1.1.1.40), which was induced by growth with DL-malate. However, none of these preparations metabolized D-malate. Intact cells of *Pseudomonas* 2,5 oxidize exogeneous D-malate only slowly; whereas the fluorescent organism *Pseudomonas* 3,5, which metabolizes alkyl-substituted phenols by similar reactions (Hopper & Chapman, 1971), also oxidizes exogenous D-malate much faster than the former organism. A cell-free system that oxidized D-malate to completion has been reconstituted from disrupted cells of *Pseudomonas* 3,5 (Hopper, Chapman & Dagley, 1970). In addition to soluble extract, the active system required 'fine particles' from the fragmented cellular membranes and also $NADP^+$; however, the initial reactions involved in D-malate metabolism were not elucidated. These reactions also remain obscure for *Pseudomonas* 2,5.

Intact cells and cell extracts of *Pseudomonas* 2,5 that degrade gentisate also catalyse homologous reactions for gentisates that bear alkyl substituents at C-3 and C-4 of the nucleus. These observations suggest that individual enzymes of the sequence possess broad substrate specificities, and this would enable the organisms to metabolize a wide range of natural products by making use of one and the same battery of enzymes. Thus, when *Pseudomonas* 2,5 was grown at the expense of any one of a series of alkyl-substituted phenols, the ability was acquired to oxidize all the others; moreover, the fact that cells grown with succinate could oxidize various

gentisic acids further suggests that the enzymes of the gentisate pathway are constitutive in this organism. In the apparent broadness of the substrate specificities of the enzymes it employs to degrade the benzene nucleus by the gentisate pathway, *Pseudomonas* 2,5 resembles those pseudomonads that oxidize catechols by the *meta*-fission pathway and can also tolerate the introduction of alkyl groups into their substrates (Bayly, Dagley & Gibson, 1966; Bayly & Dagley, 1969). These enzymes differ from those that are used by *Pseudomonas putida* to degrade protocatechuate (Ornston, 1966*a*) and catechol (Ornston, 1966*b*), which are highly substrate-specific. However, firm decisions about the specificities of the enzymes of *Pseudomonas* 2,5 cannot be made until these enzymes have been rigorously purified. Meanwhile, in support of the view that one battery of enzymes can catalyse the degradation of a range of substrates, we partially purified an oxygenase and showed that it was able to attack four alkyl-substituted gentisates readily, in addition to unsubstituted gentisate (Table 5). Further, the ring-fission products of these gentisates were also readily attacked by a maleylpyruvase that was separated from other enzymes by chromatography on a Sephadex G-150 column. The combined action of two broadly specific enzymes, gentisate oxygenase and maleylpyruvase, would also explain the conversion of 1,4-dihydroxy-2-naphthoate into phthalate and pyruvate by ammonium sulphate-treated extracts of *Pseudomonas* 2,5 grown with *m*-cresol (Scheme 1). The gentisate oxygenase of *Pseudomonas* 3,5 differs from that of *Pseudomonas* 2,5 insofar as it shows much greater variation in rates of attack on different gentisates (Table 5); thus gentisates substituted with a methyl group at C-4 were poorly oxidized. This observation may be related to the fact that this organism does not appear to encounter 4-methylgentisates during metabolism. *Pseudomonas* 2,5, which readily oxidizes 4-methylgentisate, also converts 2,5-xylenol into 3-hydroxy-4-methylbenzoate and 4-methylgentisate, so that the formation of these compounds could only be demonstrated when cells were treated with $\alpha\alpha'$-bipyridyl in order to inhibit the further oxidation of 2,5-xylenol (Hopper & Chapman, 1971). By contrast, *Pseudomonas* 3,5 did not oxidize 2,5-xylenol beyond the stage of 3-hydroxy-4-methylbenzoate, and it was therefore possible to isolate and characterize this compound without adding $\alpha\alpha'$-bipyridyl. Hopper & Chapman (1971) also found that *Pseudomonas* 3,5 could utilize neither 2,5-xylenol nor 2,3,5-trimethylphenol as substrate for growth. It therefore appears that in *Pseudomonas* 3,5, the enzyme that converts a 3-hydroxybenzoate into a gentisate cannot catalyse the required hydroxylation when C-6 bears a methyl group, and in consequence a 4-methylgentisate is never encountered in this metabolic pathway.

This work was supported in part by Grants AI 07656 and AM 13533 of the U.S. Public Health Service.

## REFERENCES

Barker, H. A. (1962). *Biochem. Prep.* **9**, 25.
Barker, H. A. & Blair, A. H. (1962). *Biochem. Prep.* **9**, 21.
Bayly, R. C. & Dagley, S. (1969). *Biochem. J.* **111**, 303.
Bayly, R. C., Dagley, S. & Gibson, D. T. (1966). *Biochem. J.* **101**, 293.
Braun, D. & Geenen, H. (1962). *J. Chromat.* **7**, 56.
Byrne, G. A. (1965). *J. Chromat.* **20**, 528.
Calvo, J. M., Kalyanpur, M. G. & Stevens, C. M. (1962). *Biochemistry, Easton*, **1**, 1157.
Chapman, P. J. & Hopper, D. J. (1968). *Biochem. J.* **110**, 491.
Cheftel, R. I., Munier, R. & Macheboeuf, M. (1951). *Bull. Soc. Chim. biol.* **33**, 840.
Cooper, R. A. & Kornberg, H. L. (1964). *Biochem. J.* **91**, 82.
Couper, M., Kibler, C. J. & Lutz, R. E. (1941). *J. Am. chem. Soc.* **63**, 2.
Croxall, W. J., Sowa, F. J. & Nieuwland, J. A. (1937). *J. org. Chem.* **2**, 253.
Dagley, S., Chapman, P. J. & Gibson, D. T. (1965). *Biochem. J.* **97**, 643.
Dagley, S., Geary, P. J. & Wood, J. M. (1968) *Biochem. J.* **109**, 559.
Dansette, P. & Azerad, R. (1970). *Biochem. biophys. Res. Commun.* **40**, 1090.
Desai, R. B. & Sethna, S. (1951). *J. Indian chem. Soc.* **28**, 213.
Fieser, L. F. & Lothrop, W. C. (1936). *J. Am. chem. Soc.* **58**, 749.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Heilbron, I. & Bunbury, H. M. (1953). *Dictionary of Organic Compounds*, 3rd ed., vol. 4, p. 192. New York: Oxford University Press.
Hickinbottom, W. J. & Garwood, R. F. (1956). In *Chemistry of Carbon Compounds*, vol. 3B, p. 843. Ed. by Rodd, E. H. Amsterdam: Elsevier Publishing Co.
Homeyer, A. H. & Wallingford, V. H. (1942). *J. Am. chem. Soc.* **64**, 798.
Hopper, D. J. & Chapman, P. J. (1971). *Biochem. J.* **122**, 19.
Hopper, D. J., Chapman, P. J. & Dagley, S. (1968). *Biochem J.* **110**, 798.
Hopper, D. J , Chapman, P. J. & Dagley, S. (1970). *J. Bact.* **104**, 1197.
Isherwood, F. A. & Hanes, C. S. (1953). *Biochem. J.* **55**, 824.
Inouye, H. (1954). *Pharm. Bull., Tokyo*, **2**, 359.
Jones, J. I. (1958). *Chemy Ind.* p. 228.
Jungwirth, C., Gross, S. R., Margolin, P. & Umbarger, H. E. (1963). *Biochemistry, Easton*, **2**, 1.
Kalbe, H. (1954). *Hoppe-Seyler's Z. physiol. Chem.* **297**, 19.
Katsuki, H., Ariga, N., Katsuki, F., Nagai, J., Egashira, S. & Tanaka, S. (1962). *Biochim. biophys. Acta*, **56**, 545.

Koegel, R. J., McCallum, R. A., Greenstein, J. P., Winitz, M. & Birnbaum, S. M. (1957). *Ann. N.Y. Acad. Sci.* **69**, 94.

Krebs, H. A. & Eggleston, N. N. (1943). *Biochem. J.* **37**, 334.

Lack, L. (1959). *Biochim. biophys. Acta*, **34**, 117.

Ornston, L. N. (1966*a*). *J. biol. Chem.* **241**, 3787.

Ornston, L. N. (1966*b*). *J. biol. Chem.* **241**, 3795.

Pastuska, G. (1961). *Z. analyt. Chem.* **179**, 355.

Rabin, R., Salamon, I. I., Bleiweis, A. S., Carlin, J. & Ajl, S. J. (1968). *Biochemistry, Easton*, **7**, 377.

Robins, D. J., Campbell, I. M. & Bentley, R. (1970). *Biochem. biophys. Res. Commun.* **39**, 1081.

Sai, T. (1968). *Agric. biol. Chem, Tokyo*, **32**, 522.

Shaw, D. A., Borkenhagen, L. F. & Talalay, P. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 837.

Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 1. London: W. Heinemann (Medical Books) Ltd.

Strassman, M. & Ceci, L. N. (1963). *J. biol. Chem.* **238**, 2445.

Swim, H. E. & Utter, M. F. (1957). In *Methods in Enzymology*, vol. 4, p. 584. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Updegraff, I. H. & Cassidy, H. G. (1949). *J. Am. chem. Soc.* **71**, 407.

von der Mühll, P. A., Settimj, G., Weber, H. & Arigoni, D. (1965). *Chimia*, **18**, 595.