JOURNAL OF BACTERIOLOGY, Mar. 1975, p. 794-799
Copyright © 1975 American Society for Microbiology
Vol. 121, No. 3
Printed in U.S.A.

Purification and Properties of Gentisate 1,2-Dioxygenase from *Moraxella osloensis*

RONALD L. CRAWFORD, SCOT W. HUTTON, AND PETER J. CHAPMAN*

Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55101

Received for publication 1 November 1974

Gentisate:oxygen 1,2-oxidoreductase (decyclizing) (EC 1.13.11.4; gentisate 1,2-dioxygenase) from *Moraxella osloensis* was purified to homogeneity as shown by polyacrylamide gel electrophoresis. The enzyme has a molecular weight of about 154,000 and gives rise to subunits of molecular weight 40,000 in the presence of sodium dodecyl sulfate. Gentisate 1,2-dioxygenase showed broad substrate specificity and attacked a range of halogen- and alkyl-substituted gentisic acids. Maleylpyruvate, the product formed from gentisate, was degraded by cell extracts supplemented with reduced glutathione, but substituted maleylpyruvates were not attacked under these conditions.

Most of the benzene ring-fission dioxygenases so far investigated are restricted in their substrate specificities to oxidation of 1,2- or 1,4-dihydric phenols (4, 6). In the latter category, substrates include homogentisic acid (5), which bacteria degrade by reactions similar to those in mammalian liver (18), and gentisic acid for which two metabolic routes have been described. Thus, some species of *Pseudomonas* degrade gentisate to pyruvate and fumarate by hydrolysis of fumarylpyruvate, formed by isomerization of maleylpyruvate, the ring-fission product of gentisate 1,2-dioxygenase (19, 25). In other species isomerization does not occur and maleylpyruvate is hydrolyzed directly to give pyruvate and maleate (14, 15). Gentisate 1,2-dioxygenase (EC 1.13.11.4, formerly EC 1.13.1.4) was purified from species of *Pseudomonas* by Lack (19) and Sugiyama et al. (24) to a degree sufficient to enable a requirement for Fe^{2+} ions to be demonstrated and to show that homogentisate, catechol, protocatechuate, and 2,3-dihydroxybenzoate were not attacked. However, further purification of the enzyme was hindered by its lability to heat and loss of activity on storage (24). We now report the purification of gentisate 1,2-dioxygenase from *Moraxella osloensis*. The enzyme from this source was heat stable and could be purified to homogeneity, as shown when examined by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Organism. The bacterium used throughout this investigation was isolated from soil in St. Paul by selective enrichment with 2-methoxybenzoic acid (o-anisic acid) as a sole source of carbon for growth. It is a gram-negative, immotile, oxidase-positive coccobacillus, and was identified as a strain (OA3) of *M. osloensis* using the transformation assay of Juni (16). It is of interest that oxidase-positive moraxellas have not been isolated previously from soil (2, 16).

Growth and cultivation of organism. *M. osloensis* OA3 was grown in a medium that contained, per liter of deionized water: $K_2HPO_4 \cdot 3H_2O$, 4.25 g; $NaH_2PO_4 \cdot H_2O$, 1.0 g; NH_4Cl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.012 g; $MnSO_4 \cdot H_2O$, 0.003 g; $ZnSO_4 \cdot 7H_2O$, 0.003 g; $CoSO_4$, 0.001 g; nitriloacetic acid, 0.01 g; and 2-hydroxybenzoic acid (salicylic acid), 1.0 g. Concentrated solutions of salicylic acid, neutralized with NaOH, were autoclaved separately and added to the cooled, sterile mineral salts buffer. Cultures were grown at 35 to 37 C with forced aeration in 20-liter carboys containing 16 liters of growth medium. Each carboy was inoculated with one 48-h culture of *M. osloensis* OA3 grown in 1 liter of the mineral salts medium supplemented with 0.5 g of yeast extract (Difco). After growth for 18 to 20 h after inoculation, 16 g of salicylic was added; cells harvested 3 to 5 h later were washed once with 0.1 M Na-K phosphate buffer, pH 7.4. One carboy yielded 45 to 50 g of cell paste. Stock cultures of *M. osloensis* OA3 were maintained on nutrient agar slants, stored at 4 C under Parafilm (American Can Co.), and subcultured monthly. Cells from one slant served to seed an inoculum culture of 1 liter.

Preparation of cell extracts. The organism was quite resistant to disruption procedures. Thus, prolonged sonic treatment gave low yields of broken cells; however, the French pressure cell (American Instrument Co., Silver Spring, Md.) was more effective. Cell paste was suspended in 2 volumes of 0.1 M Na-K phosphate buffer (pH 7.4) containing 25% glycerol, and the suspension was passed twice through a French pressure cell at >10,000 lb/in^2 applied with a Carver laboratory press. Deoxyribonuclease and ribo-

nuclease (Sigma Chemical Co., St. Louis, Mo.) were added to decrease viscosity, and the extract, after clarification by centrifuging at 30,000 × g for 30 min, contained about 20 mg of protein per ml.

Enzyme assay. It was necessary to activate all enzyme preparations, except crude extracts, with Fe^{2+} ions prior to assay. Accordingly, 0.25 ml of 0.01 M ferrous ammonium sulfate was incubated with each 1 ml of enzyme solution for 20 min at 25 C before use. Assay mixtures contained 0.15 μmol of gentisic acid in 3 ml of 0.1 M Na-K phosphate buffer (pH 7.4) and the reaction, started by addition of enzyme, was followed by measuring the increase in absorbance at 334 nm. A value of ϵ equal to 10,800 was used to calculate enzyme activities, one unit being the amount of enzyme that produces 1 μmol of maleylpyruvate per min.

Purification of gentisate 1,2-dioxygenase. Except when stated otherwise, all procedures were performed at 0 to 5 C. Phosphate buffer refers to Na-K phosphate buffer, pH 7.4, containing 25% (vol/vol) glycerol. This enzyme, like protocatechuate 4,5-oxygenase (27), is protected by the presence of glycerol during purification.

Step 1. Crude extract was held at 60 C for 7 min and then rapidly cooled in an ice bath, and the precipitate was removed by centrifuging at 30,000 × g for 20 min.

Step 2. The solution was brought to 70% saturation by the addition of a neutralized solution of ammonium sulfate saturated at 4 C. The precipitated protein was collected by centrifugation, redissolved in 30 ml of 0.1 M phosphate buffer, applied to a column of G-150 Sephadex (5 by 55 cm) pre-equilibrated with 0.1 M phosphate buffer and then washed with the same buffer. Samples of 9 ml were collected, and the fractions (36 to 45) that contained the enzyme were pooled and brought to 70% saturation with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 2 to 4 ml of 0.01 M phosphate buffer.

Step 3. After dialysis against three changes of 0.01 M phosphate buffer, the enzyme was applied to a column (2 by 34 cm) of triethylaminoethyl-cellulose that had been equilibrated with 0.01 M phosphate buffer. A linear gradient of 0 to 0.5 M NaCl in 0.01 M phosphate buffer (25% glycerol) was applied, and gentisate 1,2-dioxygenase was eluted at 0.28 to 0.31 M NaCl. Peak fractions were pooled and brought to 70% saturation with ammonium sulfate, and the precipitate was collected on the centrifuge and dissolved in 1 to 2 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0, containing 25% glycerol.

Step 4. The enzyme was dialyzed against three changes of the same Tris-hydrochloride-glycerol buffer and applied to a column (2 by 10 cm) of diethylaminoethyl-cellulose which had been equilibrated with this buffer. A linear gradient of 0 to 0.5 M NaCl in Tris-hydrochloride-glycerol buffer was applied, and gentisate 1,2-dioxygenase was eluted at 0.30 to 0.32 M NaCl. Peak fractions were pooled and could be stored at −15 C for 6 months without loss of activity. However, in buffers that did not contain glycerol, the enzyme lost activity completely at 4 C within 2 weeks.

Polyacrylamide gel electrophoresis. Details of the procedures used to examine the purified enzyme by the method of Davis (9), and also by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26), were described in a previous communication (20). The electrophoretic mobility of each protein, expressed as a ratio of the distance of migration of the protein to that of dye, was corrected for swelling of gels during destaining as described by Weber and Osborn (26).

Molecular weight. The molecular weight of gentisate 1,2-dioxygenase was determined by gel filtration as described in the manual provided by Bio-Rad Laboratories, Richmond, Calif. A column of Bio-Gel A-1.5m (0.5 by 115.5 cm) was used, having a bed volume, V_t, of 23.1 cm^3 and void volume, V_o, of 8.8 cm^3. The column was equilibrated with 0.1 M Na-K phosphate buffer, pH 7.4, and an elution volume, V_e, was determined for each protein (standards and enzyme) by applying 5 mg of protein in 0.2 ml of phosphate buffer, followed by elution with phosphate buffer at a flow rate of 2 ml/h. Fractions of 0.5 ml were collected and protein contents were determined (12). Ratios of V_e/V_0 (= K_{av}) were plotted versus molecular weight on a logarithmic scale.

The following alkyl-substituted gentisic acids were prepared as previously described: 3-methylgentisic acid, 4-methylgentisic acid, 3-ethylgentisic acid, and 3,4-dimethylgentisic acid (13); 6-methylgentisic acid (15). Several halogenated gentisic acids were also prepared by persulfate oxidation (13) of the corresponding halogenated salicylic acids. Thus, 3-fluorogentisic acid (melting point [mp] 218 to 220 C, decomposition) was obtained from 3-fluorosalicylic acid; 4-fluorogentisic acid (mp 220 to 223 C, decomposition) was from 4-fluorosalicylic acid (7); 3-chlorogentisic acid (mp 219 to 220 C, decomposition) from 3-chlorosalicylic acid (21); and 4-chlorogentisic acid (mp 222 to 223 C, decomposition) was from 4-chlorosalicylic acid (3). Halogenated salicylic acids were prepared as follows. 3-Fluorosalicylic acid was obtained by high-pressure carboxylation of the sodium salt of 2-fluorophenol (11). 4-Fluorosalicylic acid, mp 187 to 188 C (10), was obtained by a similar reaction, the product being separated from a minor quantity of 6-fluorosalicylic acid, mp 163 to 165 C, by fractional crystallization. Silver oxide oxidation (22) of 3-chlorosalicylaldehyde, prepared from 2-chlorophenol by a Reimer-Tiemann reaction (8), gave 3-chlorosalicylic acid, mp 181 to 182 C (23). 4-Chlorosalicylic acid (mp 210 to 212 C) was prepared from 2-amino-4-chlorobenzoic acid (ROC/IC Corp., Sun Valley, Calif.) by diazotization and boiling (10). 3-Bromogentisic acid (mp 249 to 251 decomposition) (17) was obtained by direct bromination of gentisic acid. All other chemicals were available from commercial sources.

RESULTS AND DISCUSSION

Molecular weight and subunit structure. Gentisate 1,2-dioxygenase, purified 31-fold by

the procedure of Table 1, gave a single band that stained for protein when examined by polyacrylamide gel electrophoresis at pH 8.9 (9). From the results of gel filtration on a standardized column of Bio Gel A-1.5m, the molecular weight of the dioxygenase was estimated to be about 154,000 (Fig. 1). The subunit composition of the purified enzyme was determined by disc-gel electrophoresis analysis of the enzyme in the presence of sodium dodecyl sulfate (26) before and after reduction with mercaptoethanol as previously described (20). In both experiments a single band was obtained, corresponding to a molecular weight of 40,000 when comparison was made with the migration of other proteins of known molecular weight (Fig. 2). Thus, gentisate 1,2-dioxygenase appears to be a tetramer of subunits with identical molecular weight. Adachi et al. (1) gave 380,000 for the molecular weight of homogentisate 1,2-dioxygenase (EC 1.13.11.5) from *Pseudomonas fluorescens;* subunit composition was not reported.

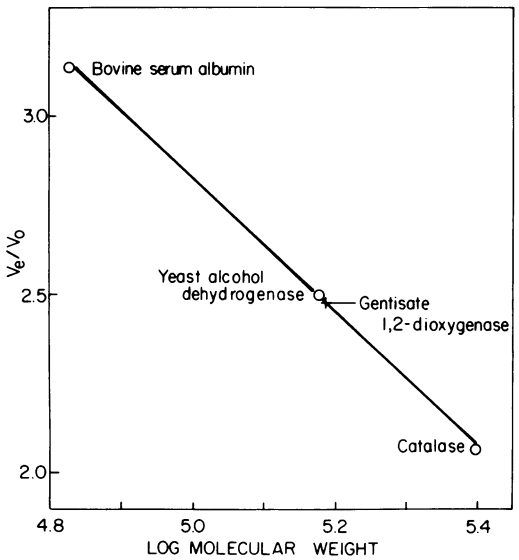


FIG. 1. *Molecular weight of purified gentisate 1,2-dioxygenase as determined by Bio Gel (A-1.5m) gel filtration. The ratio of elution volume, V_e, to void volume, V_o, was determined for the enzyme and for proteins of known molecular weight.*

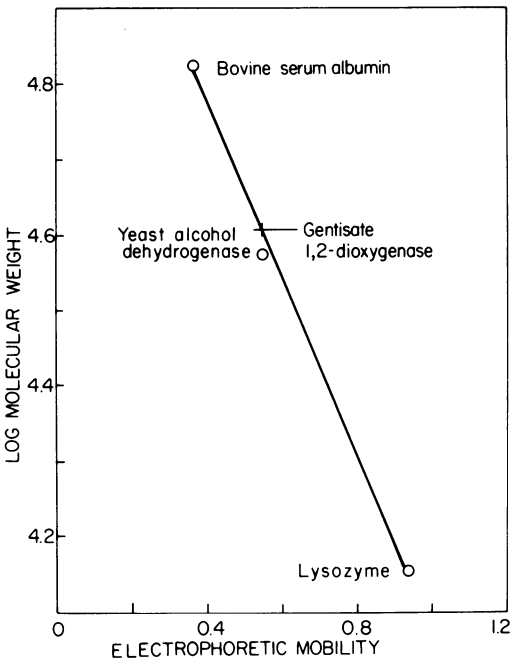


FIG. 2. *Molecular weight of the subunits of gentisate 1,2-dioxygenase as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.*

Substrate specificity of gentisate 1,2-dioxygenase. The ability of 10 substituted gentisic acids to serve as substrates was examined by means of Warburg respirometry. In view of the quantity of enzyme required in these experiments, extract from step 2 of Table 1 was used (purified 5.5-fold); this preparation was free from maleylpyruvate isomerase and fumarylpyruvate hydrolase (see Fig. 4). Each flask contained 0.2 ml of 5 N NaOH in the center well and 10 mg of Fe^{2+}-activated enzyme protein in

TABLE 1. *Purification of gentisate 1,2-dioxygenase*

Step	Protein (mg)	Units[a]	Sp act[b]	Purification (fold)	Recovery (%)
Crude extract	1,363	523.4	0.384	1	100
1. Heat treatment	798	664.1	0.834	2.2	127
2. Sephadex G-150	81	178.2	2.2	5.5	34
3. TEAE-cellulose[c]	20	130.0	6.5	16.9	25
4. DEAE-cellulose[c]	10	120.0	12.0	31.3	23

[a] One unit is the amount of enzyme that catalyzes the formation of 1 μmol of maleylpyruvate per min.
[b] Units per milligram of protein.
[c] TEAE, Triethylaminoethyl; DEAE, diethylaminoethyl.

2.3 ml of 0.1 M Na-K phosphate buffer, (pH 7.4) in the main compartment. Reactions were started by addition of 5 μmol of substrate from the side arm, and initial rates of uptake of oxygen were measured for the first 5 min of the reaction. The enzyme attacked 3-ethylgentisate, 3- and 4-methylgentisates, and all the halogen-substituted gentisates investigated; 6-methylgentisate and 3,4-dimethylgentisate were not attacked (Table 2). Flask contents from this experiment were cooled to 0 C, deproteinized by adding 0.2 ml of 2 N sulfuric acid, and centrifuged, and each clear supernatant solution was made up to 10 ml with 1 M Tris-hydrochloride buffer, pH 8.0. Absorption spectra similar to that reported for maleylpyruvate by Lack (19) were observed; molar extinction coefficients (ϵ) at λ_{max} were calculated by assuming that 1 mol of ring-fission product was formed with consumption of 1 mol of oxygen (Table 3). Values for the ring-fission products from 3- and 4-fluorogentisates and 4-chlorogentisates are not recorded since these compounds decomposed during the isolation procedure.

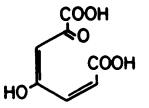
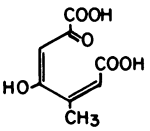
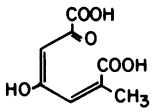
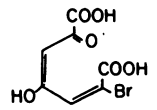
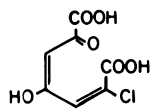
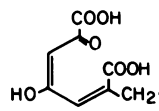
Kinetic properties. The enzyme showed a broad pH optimum between pH 7.0 and 8.5, with maximal activity near pH 7.5. At this pH, activity in Tris-hydrochloride buffer was almost twice that in phosphate buffer. A Lineweaver-Burk plot was linear and gave an apparent K_m of 7.1×10^{-6} M for gentisate, determined with oxygen at the pressure in the atmosphere. We confirmed the observation of Sugiyama et al. (24) that *o*-phenanthroline and 2,2'-dipyridyl are powerful inhibitors (>90% inhibition at 0.05 mM); further, Tiron (4,5-dihydroxy-*m*-benzene disulfonic acid, sodium salt), which is a chelating agent for trivalent iron, was without effect at this concentration and inhibited by 23% at 0.17 mM.

TABLE 2. *Rates of oxidation of gentisate, and substituted gentisates, by gentisate 1,2-dioxygenase*

Substrate	Initial[a] rate	Relative rate
Gentisate	0.38	1.00
4-Fluorogentisate	0.33	0.88
3-Methylgentisate	0.24	0.63
3-Ethylgentisate	0.18	0.47
3-Chlorogentisate	0.17	0.45
3-Fluorogentisate	0.13	0.34
4-Methylgentisate	0.12	0.32
4-Chlorogentisate	0.11	0.29
3-Bromogentisate	0.09	0.24
6-Methylgentisate	ND[b]	
3,4-Dimethylgentisate	ND	

[a] Micromoles of O_2 per minute.
[b] ND, Not detectable.

TABLE 3. *Ultraviolet spectral properties of solutions (1 M Tris-hydrochloride buffer, pH 8.0) of halogen- and alkyl-substituted maleylpyruvates*

PRODUCT	λ_{max} (nm)	ϵ ($\times 10^{-4}$)
	334	1.08
	306	0.78
	332	1.21
	340	1.13
	340	1.54
	333	1.47

Metabolism of maleylpyruvate. Extracts of *M. osloensis* contained an isomerase dependent on reduced glutathione; this was shown in the following experiment. Crude extract was brought to 70% saturation with ammonium sulfate; the precipitated protein was collected by centrifugation, dissolved in 0.05 M Na-K phosphate buffer, pH 7.1, and dialyzed for 3 h at 5 C against two changes of the same buffer. Gentisate 1,2-dioxygenase was then activated by incubation with ferrous ammonium sulfate, and 0.3 ml of cell extract (0.6 mg of protein) was added to each of three cuvettes containing 0.125 μmol of gentisate in 3 ml of 0.02 M Na-K phosphate buffer, pH 7.1. The absorbance at 330 nm, due to formation of maleylpyruvate, increased immediately on addition of cell extract and reached a maximum value in each cuvette

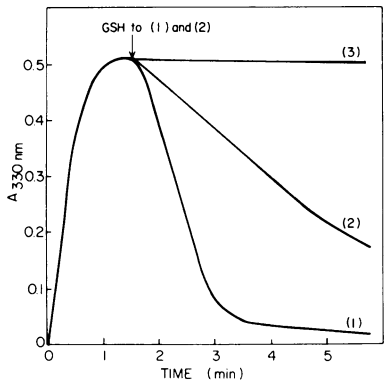


FIG. 3. *Dependence of maleylpyruvate degradation on additions of reduced glutathione. Conversion of gentisate to maleylpyruvate was monitored by the increase in absorbance at 330 nm, and at 1.5 min 50 μM GSH was added to cuvette 1, and 5 μM GSH was added to 2. No addition was made to cuvette 3.*

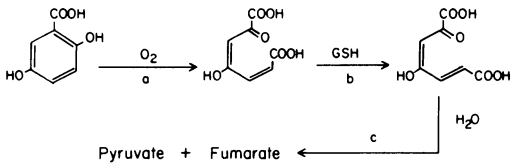


FIG. 4. *Degradation of gentisate by M. osloensis grown with salicylate. Enzymes referred to in the text are: (a) gentisate 1,2-dioxygenase; (b) maleylpyruvate isomerase; (c) fumaryl-pyruvate hydrolase.*

after 1.5 min. Additions of reduced glutathione to two cuvettes resulted in a decrease in absorbance, which was not observed for the third curvette to which no addition was made (Fig. 3). This experiment was repeated using 3- and 4-fluorogentisates and 3- and 4-chlorogentisates as substrates. Ring-fission compounds accumulated, but in no case were they metabolized on addition of reduced glutathione. It therefore appears that these compounds do not serve as substrates for maleylpyruvate isomerase (Fig. 4). By contrast, extracts of the organism *Pseudomonas* 2,5 (British NCIB 9867) of Hopper et al. (15) were found to degrade halogen-substituted maleylpyruvate (unpublished observations). This pseudomonad, like *M. osloensis* appears to possess a gentisate 1,2-dioxygenase of broad specificity but metabolizes maleylpyruvate by hydrolytic fission without prior isomerization to fumarylpyruvate (15).

ACKNOWLEDGMENTS

We thank Eliot Juni for providing a culture of *M. osloensis* E55, Terry Sayther for assistance in chemical syntheses, and Stanley Dagley for helpful discussions. We also thank the 3M Company, St. Paul, Minn., for providing facilities for high-pressure reactions, and Manfred Riechert who used them to carry out carboxylations involved in chemical synthesis.

This investigation was supported by Public Health Service grant ES A1 00678 from the National Institute of Environmental Health Sciences, and by grant GB 44228 from the National Science Foundation.

LITERATURE CITED

1. Adachi, K., Y. Iwayama, H. Tanioka, and Y. Takeda. 1966. Purification and properties of homogentisate oxygenase from *Pseudomonas fluorescens*. Biochim. Biophys. Acta **118**:88-97.
2. Baumann, P. 1968. Isolation of *Acinetobacter* from soil and water. J. Bacteriol **96**:39-42.
3. Bhattacharya, S. C., and D. E. Seymour. 1950. 4-Aminosalicylic acid and its derivatives. Part II. The synthesis of 4-amino-2:5- and 4-amino-2:3-dihydroxybenzoic acid. J. Chem. Soc. 1139-1140.
4. Chapman, P. J. 1972. An outline of reaction sequences used for the bacterial degradation of phenol compounds, p. 17-55. *In* Degradation of synthetic organic molecules in the biosphere. Printing and Publishing Office, National Academy of Sciences, Washington, D.C.
5. Chapman, P. J., and S. Dagley. 1962. Oxidation of homogentisic acid by cell-free extracts of a vibrio. J. Gen. Microbiol. **28**:251-256.
6. Dagley, S. 1971. Catabolism of aromatic compounds by microorganisms. Adv. Microb. Physiol. **6**:1-46.
7. Danek, O., and S. Nouzova. 1968. Preparation of fluorine containing analogs of some pharmacologically active substances. Collect. Czech. Chem. Commun. **33**:425-430.
8. Davies, W., and L. Rubenstein. 1923. Substitution in vicinal trisubstituted benzene derivatives. Part II. J. Chem. Soc. 2839-2852.
9. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404-427.
10. Dunn, G. E., and T. L. Penner. 1967. Effect of intramolecular hydrogen bonding on the relative acidities of substituted salicylic acids in benzene solution. Can. J. Chem. **45**:1699-1706.
11. Ferguson, L. N., J. C. Reid, and M. Calvin. 1946. Some new fluorinated phenolic aldehydes and acids. J. Am. Chem. Soc. **68**:2502-2504.
12. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret method. J. Biol. Chem. **177**:751-766.
13. Hopper, D. J., and P. J. Chapman. 1971. Gentisic acid and its 3- and 4-methyl-substituted homologues as intermediates in the bacterial degradation of *m*-cresol, 3,5-xylenol and 2,5-xylenol. Biochem. J. **122**:19-28.
14. Hopper, D. J., P. J. Chapman, and S. Dagley. 1968. Enzymic formation of D-malate. Biochem. J. **110**:798-800.
15. Hopper, D. J., P. J. Chapman, and S. Dagley. 1971. The enzymic degradation of alkyl-substituted gentisates, maleates and malates. Biochem. J. **122**:29-40.
16. Juni, E. 1974. Simple genetic transformation assay for rapid diagnosis of *Moraxella osloensis*. Appl. Microbiol. **27**:16-24.
17. Kloetzel, M. C., and B. Y. Abadir. 1955. Synthetic analogs of cortical hormones. Part II. 3-Substituted α-2,5-trihydroxyacetophenone derivatives. J. Am. Chem. Soc. **77**:3823-3826.
18. Knox, W. E., and S. W. Edwards. 1955. The properties of maleylacetoacetate, the initial product of homogentisate oxidation in liver. J. Biol. Chem. **216**:489-498.

19. Lack, L. 1959. The enzymic oxidation of gentisic acid. Biochim. Biophys. Acta **34**:117–123.
20. Leung, P.-T., P. J. Chapman, and S. Dagley. 1974. Purification and properties of 4-hydroxy-2-ketopimelate aldolase. J. Bacteriol. **20**:168–172.
21. Lightowler, J. E., and H. J. Rylance. 1963. Substituted dihydroxybenzoic acids as possible antiinflammatory agents. J. Pharm. Pharmacol. **15**:633–638.
22. Pearl, I. A. 1946. Reactions of vanillin and its derived compounds. I. The reaction of vanillin with silver oxide. J. Am. Chem. Soc. **68**:429–432.
23. Shackelford, J. M. 1961. Ultraviolet spectra of 1,2,3-trisubstituted aromatic compounds. J. Org. Chem. **26**:4908–4911.
24. Sugiyama, S., K. Yano, and K. Arima. 1960. Metabolism of aromatic compounds by microbes. Part VIII. Further studies of gentisic acid oxidase. Bull. Agr. Chem. Soc. Jap. **24**:249–254.
25. Tanaka, H. S., S. Sugiyama, K. Yano, and K. Arima. 1957. Isolation of fumarylpyruvic acid as an intermediate of the gentisic acid oxidation by *Pseudomonas ovalis* var. S-5. Bull. Agr. Chem. Soc. Jap. **21**:67–68.
26. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412,
27. Zabinski, R., E. Muenck, P. M. Champion, and J. M. Wood. 1972. Kinetic and Mossbauer studies on the mechanism of protocatechuate 4,5-oxygenase. Biochemistry **11**:3212–3219.