Bacterial Attack on Phenolic Ethers

AN ENZYME SYSTEM DEMETHYLATING VANILLIC ACID

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1. A cell-free system from *Pseudomonas fluorescens* catalysed the oxidative demethylation and subsequent ring-cleavage of vanillate, with uptake of 2.5 moles of oxygen/mole of substrate. 2. Demethylation involved absorption of 0.5 mole of oxygen/mole, and required reduced glutathione (GSH) and nucleotide (probably NADPH) as cofactors, with further possible requirements, the natures of which are discussed. 3. Incomplete evidence suggested that the aromatic ring was opened via protocatechuate and the appropriate oxygenase, with absorption of 1 mole of oxygen/mole of substrate, eventually yielding β -oxoadipate. 4. The methyl group was removed sequentially as formaldehyde, formate and carbon dioxide, the steps catalysed respectively by formaldehyde dehydrogenase, which required GSH and NAD⁺, and formate dehydrogenase. Each enzyme was cytochrome-linked and accounted for absorption of 0.5 mole of oxygen/mole of substrate. 5. All enzymes except formate dehydrogenase, which was a cell-wall enzyme, resided in the soluble fraction of the extract. The demethylase could not be resolved because of unknown cofactor requirements.

The interpretation of the results of bacterial attack on lignin is difficult because of the chemical complexity of this material. To obtain essential background information for this difficult problem we have examined the bacterial degradation of substances that could be products of lignin catabolism or that, by dehydrogenation and condensation reactions, could be converted into lignin in plants.

We now present, for the first time, observations on demethylation processes by partly resolved bacterial enzymes. Whereas Axelrod (1956) and Brodie *et al.* (1955) demonstrated salient features of the enzymic demethylation of methoxyphenyl derivatives in mammalian tissues, no detailed information has been reported on the process in micro-organisms; although, for example, Henderson (1957, 1961) showed that demethylated products arise from the attack by fungal cells on methoxybenzoic acids.

Ferulate, which can function as a lignin precursor (Siegel, 1954; Ishikawa & Takaichi, 1955), was oxidized by a strain of *Pseudomonas fluorescens* isolated in this Laboratory to vanillate (4-hydroxy-3-methoxybenzoate), which in turn was demethylated before further oxidation, probably via protocatechuate, to products involved in normal

intermediary metabolism. Cell-free extracts of the bacteria were used to determine intermediates and cofactors of the demethylation process.

EXPERIMENTAL

Organism. A strain of *Pseudomonas fluorescens* (strain T) isolated by conventional enrichment technique from the aqueous liquor of stored coal tar, with the medium described below, was used.

Media. Semi-defined medium (adjusted to pH 7.2–7.5) for the culture of adapted cells consisted of (g./l.): KH₂PO₄, 0.1; K₂HPO₄, 0.4; (NH₄)₂SO₄, 1.0; MgSO₄·7H₂O, 0.01; Oxoid yeast extract, 0.1; aromatic substrate, 1.5; trace-elements solution (Barnett & Ingram, 1955), 10 ml./l.; made up in tap water. Ferulic acid was sterilized by filtration of a solution of its sodium salt, which was added aseptically to the autoclaved salts-yeast-extract solution. For routine maintenance of cultures this medium was solidified by the addition of 1.5% of agar.

For the culture of unadapted cells the aromatic substrate in the above medium was replaced by sodium gluconate (10 g.), sodium acetate (20 g.) or sodium butyrate (1.5 g.).

Cell growth. For the production of quantities of adapted and unadapted cells, the required medium was prepared in 4l. and 20l. amounts in a bolt-head flask of suitable size fitted with: (a) an inlet tube through which air, sterilized previously by passage over oil-soaked glass beads, through 50% (v/v) H₂SO₄ and finally a sterile 30 cm. × 2.5 cm. cotton-wool filter, could be introduced; (b) a Vibromischer reciprocating electrical drive (Chemap A.G., Zurich, Switzerland) with a type S.U.B. pump attached, to ensure

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adequate aeration and foam control; (c) an outlet tube plugged with cotton wool. Growth from a 24 hr. slope culture of the organism was transferred in sterile water suspension to sterile medium (500 ml.) contained in a 2 l. Erlenmeyer flask and incubated with shaking for 8 hr. at 24–26°. The resulting growth served as inoculum for 4 l. and 20 l. cultures, which were harvested after 16 and 24 hr. respectively in an Alfa-Laval type 1229G continuous-flow separator at a rate of approx. 30 l./hr. The collected cells were then washed and centrifuged (17000g for 6 min.) three times with 0.9% NaCl, 0.067M-phosphate buffer, pH 7.8, or 0.1M-tris buffer, pH 7.8. The buffer used is specified in individual experiments.

Cell breakage. (a) Ultrasonic treatment. A thick slurry of cells (1 vol.) in buffer or 0.9% NaCl (1 vol.), contained in a cellulose nitrate tube (approx. 3.0 cm. diam.) and cooled in an ice-salt bath, was irradiated for 6 min. by a titanium transforming stub (step diam. 1.25 and 0.75 in.) attached to a Mullard transducer energized by 200 w at approx. 20 kc./sec. The ruptured cell suspension was centrifuged (18000g for 15 min.) to remove whole cells and then, if desired, freed of particulate material in a Spinco model L preparative ultracentrifuge (50 rotor) at 72000g for 45 min. Electron microscopy of particulate material precipitated in the second centrifugation showed it to be cell-free. Protein concentration of the particulate-free supernatant was usually about 45 mg./ml.

(b) Press treatment. This was often used to prepare more concentrated cell-free extracts, and was carried out at –20° in an X-press (a modified Hughes Press manufactured by A. B. Biox, Nacka, Sweden). For good disruption three or four passages through the orifice were necessary. A short treatment with a few crystals of deoxyribonuclease (from bovine pancreas, minimum 33% activity of crystal; Koch-Light Laboratories Ltd., Colnbrook, Bucks.) and dilution with buffer or 0.9% NaCl (0.5–1.0 vol./vol. of concentrated cell crush) was necessary to decrease the viscosity of the product sufficiently to allow removal of unbroken cells and cell debris as indicated for the ultrasonically treated cells. No account was taken of possible proteolytic activity in the deoxyribonuclease preparation. The g values and times required depended on the viscosity of the extract and its protein concentration; in the preparation of the more concentrated cell-free extracts centrifugation at 23000g for 30 min. generally sufficed for the quantitative removal of whole cells. Protein concentrations in the supernatant material with cell debris removed by further centrifugation ranged from 45 to 60 mg./ml. or more.

Estimations. Protein was estimated by the biuret method of Gornall, Bardawill & David (1949), with bovine serum albumin fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as the standard.

β -Oxadipate was estimated by the catalytic decarboxylation method of Siström & Stanier (1953).

The chromatotropic acid colour reaction was used to determine formaldehyde (after MacFadyen, 1945) either directly, or on the aldehyde trapped as semicarbazone. Aqueous formaldehyde (A.R. grade; British Drug Houses Ltd., Poole, Dorset), estimated before use by oxidation with excess of standard I_2 and back-titration with standard $Na_2S_2O_3$, served as a standard. Incubation mixtures for assay were deproteinized by heating at 100° for 5 min. with subsequent centrifugation. Assays for formaldehyde were

performed directly on 0.10 ml. of the supernatant fluids. Incubation mixtures containing a known amount of formaldehyde were deproteinized immediately after preparation, to provide controls for the slight loss of specific colour intensity due to interference from cell-extract substances that were not precipitated by the heat treatment. To minimize colour loss, all incubations were performed in the presence of a low protein concentration (about 6 mg./ml.). Formate was estimated manometrically by the action of freshly prepared formate dehydrogenase from *Escherichia coli* (Gale, 1939), in 0.067M-phosphate buffer, pH 6.0, at 37°. Desiccator-dried sodium formate was used as a standard. Dry-weight determinations on particulate material were made after bringing the sample to constant weight at 60°.

Manometric procedures. The O_2 uptake and CO_2 production were followed by standard methods in Warburg respirometers (Warburg Apparatus model V; B. Braun G.m.b.H., Melsungen, W. Germany).

Spectrophotometry. Measurements were made in 1 cm. silica cuvettes in a Uvispek H. 700 instrument (Hilger and Watts Ltd., London).

Chemicals. Most compounds used as substrates were commercial samples purified by recrystallization or distillation as required.

NAD⁺ (grade III), NADP⁺ and their reduced forms (NADPH, chemically reduced), GSH (99% pure), ADP (98–100% pure), CoA (reduced form; 85% pure) and tetrahydrofolic acid (grade III) were from Sigma Chemical Co., St Louis, Mo., U.S.A.

Cysteine, homocysteine and 2,3-dimercaptopropan-1-ol (BAL) were purchased from Koch-Light Laboratories Ltd.

Ion-exchange resins were products of Rohm and Haas, Philadelphia, Pa., U.S.A., and were cleaned by several cycles through N-NaOH and N-HCl followed by three washes in distilled water.

Calcium phosphate gel was prepared after the method of Keilin & Hartree (1938), and was aged for 3 months before use.

Ferulic acid, m.p. 174° after recrystallization from aq. 30% (v/v) ethanol, was prepared by the method of Pearl & Bayer (1951).

Isovanillic acid (3-hydroxy-4-methoxybenzoic acid) was prepared as follows. Isovanillin (2.4 g.), benzyl bromide (1.5 ml.) and anhydrous K_2CO_3 (5 g.) were refluxed with acetone (75 ml.) for 1 hr. The residue left after distilling off the acetone was treated with excess of 2N-NaOH to dissolve unchanged isovanillin. The crude benzyl derivative was extracted into ether (4 × 25 ml.) and, after removal of the solvent, was suspended in hot water (50 ml.) at 60°. Powdered $KMnO_4$ (10 g.) in water (100 ml.) was then added slowly until the permanganate colour was not discharged. The MnO_2 was filtered off and 3-benzyloxy-4-methoxybenzoic acid separated slowly after acidification (HCl) of the filtrate. The crude derivative suspended in conc. HCl (10 ml.) and acetic acid (15 ml.) was debenzylated by heating for 30 min. on a water bath. After dilution with water (25 ml.) isovanillic acid crystallized on standing. Recrystallization from water gave a product, m.p. 248–250° (authentic isovanillic acid had m.p. 247–248°). 3-Ethoxy-4-hydroxybenzoic acid, m.p. 157–159° (reported m.p. 164–165°), and o-vanillic acid (2-hydroxy-3-methoxybenzoic acid), m.p. 148–150° (authentic material + H_2O , m.p. 150°), were prepared from the corresponding aldehydes

by identical procedures. The reported and observed melting points of 3-ethoxy-4-hydroxybenzoic acid differ considerably. Unfortunately, no authentic material was available for comparison, but the properties of our material were compatible with the correct structure.

Thiazolidine-4-carboxylic acid, m.p. 196–197° (decomp.), was prepared by the method of Ratner & Clarke (1937). Thiazine-4-carboxylic acid, m.p. 204–206° (decomp.), was prepared by an identical procedure starting from homocysteine, and was recrystallized from aq. 33% (v/v) propan-2-ol. β -Oxadipic acid, m.p. 125–126° (decomp.), was synthesized by the method of Bardhan (1936).

Paper chromatography. This was carried out by descending techniques. Solvent systems used were: A, acetic acid-mesityl oxide-water (9:10:3, by vol.); B, butan-1-ol-cyclohexane-propane-1,2-diol-aq. 33% (w/v) ethylamine-morpholine-water (41:41:13:0.9:0.1:4, by vol.); C, ethanol-aq. ammonia (sp.gr. 0.88)-water (20:1:4, by vol.); D, propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.). The paper used is specified in the text. A Chromatolite narrow-range u.v. lamp was used for preliminary detection of absorptive or fluorescent substances. Phenolic compounds were located by spraying with alkaline diazotized 4-aminophenyl-2'-diethylaminoethyl sulphone (I.C.I. 5091) (Koch-Light Laboratories Ltd.). The diazo compound was prepared by mixing an equal volume of I.C.I. 5091 (1%, w/v, solution in 1.1N-HCl) with 4% (w/v) NaNO_2 at 4° and rendering alkaline after 5 min. with excess of $\text{M-Na}_2\text{CO}_3$. Diphenols were detected by spraying with 3% (w/v) FeCl_3 hydrate, adjusted to pH 1 with 2N-NaOH. Amino acids and their derivatives were located by the ninhydrin spray reagent of Cummins & Harris (1956) with subsequent heating at 100° for 3 min. to accelerate development. Incubation mixtures for examination (2.8 ml.) were deproteinized with 10% (w/v) trichloroacetic acid (0.1 vol.) followed by centrifugation. Supernatants were passed down an Amberlite IR-400 (OH^- form) resin column (14 cm. $\times$ 1 cm.), followed by a small amount of ethanol. Elution was effected with N-HCl in 70% (v/v) ethanol (about 20 ml.). Strong anions were removed from the eluate by resin (HCO_3^- form) treatment until CO_2 evolution ceased and the pH had risen to 4.0. The samples, freed of spent resin, were evaporated to dryness *in vacuo*, redissolved in 0.3 ml. of aq. 10% (v/v) propan-2-ol, spotted on to Whatman no. 4 paper and run against authentic markers in solvent A. Thiazine-4-carboxylic acid ran slightly behind homocysteine and gave a grey spot with ninhydrin spray. Formic acid was detected with ammoniacal AgNO_3 spray (0.1N- AgNO_3 , 1 vol., mixed with aq. 5N- NH_3 , 1 vol., just before use) (Brown, 1950) or with bromocresol green (bromocresol green, 0.1 g.; citric acid, 0.2 g.; ethanol, 10 ml.; water, 90 ml.) (Pike, 1960). Contrast with both reagents was heightened by subsequently heating the papers at 45–50° for 15 min. Incubation mixtures containing substrate (100 μ moles), GSH (10 μ moles), NADPH (5 μ moles) and soluble fraction (S_1) from cell extract (300 mg. of protein) in a final volume of 28 ml. were incubated at 30°. After oxidation was completed (as indicated respirometrically under parallel conditions), formate was detected as follows. Flask contents were deproteinized with $(\text{NH}_4)_2\text{SO}_4$ (6.0 g.) and 25% (v/v) H_2SO_4 (3.0 ml.) followed by centrifugation, and the supernatants were steam-distilled. Distillates (200 ml.) were brought to pH 9 (10mN-NaOH), evaporated to dryness at

100°, redissolved in 0.8 ml. of very dilute aqueous bromocresol green, and treated cautiously with Amberlite resin IR-120 (H^+ form) to remove Na^+ ions, until the pH fell to 4.0. After removal of spent resin, 0.1 ml. of aq. 33% ethylamine was added to re-establish alkalinity. Samples (2 μ l.) were then loaded on to Whatman no. 4 paper and rapidly transferred without drying to a tank held at 4° for development in solvent B. Oxo acids, run as their 2,4-dinitrophenylhydrazones, were detected either under u.v. light, or under alkaline conditions in visible light.

RESULTS

O-Demethylation of vanillate

Activity of unsupplemented bacterial extracts. It has been shown (N. J. Cartwright & A. R. W. Smith, unpublished work) that growth of organisms on ferulate gave rise to vanillate and that the cells were adapted to metabolize vanillate. Cells stored at –25° retained their activity towards vanillate for approx. 4 weeks, but cell extracts stored under the same conditions lost activity rather more rapidly. Activity towards the primary substrate

Fig. 1. Oxygen uptakes with vanillate, protocatechuate and β -oxoadipate in the presence of concentrated cell-free extract of *P. fluorescens* T. The incubation temperature was 30°, the gas phase air and the total fluid volume 2.0 ml. Each Warburg flask contained crude cell crush of *P. fluorescens* T containing soluble fraction equivalent to 52.5 mg. of protein and 7.5 mg. of particulate material, and 0.067M-phosphate or 0.1M-tris buffer, pH 7.8, to 1.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. The reaction was initiated by tipping 2 μ moles of substrate from the side arm. Controls contained no substrate. Endogenous respiration was subtracted. In phosphate buffer: $\circ$, vanillate; Δ , protocatechuate; $\square$, β -oxoadipate. In tris buffer: $\bullet$, vanillate; $\blacktriangle$, protocatechuate; $\blacksquare$, β -oxoadipate.

was not observed with cell-free extracts despite intensive experimentation involving variations of culture conditions and disruption procedures, and supplementation with a wide range of cofactors, 'sparker' compounds etc.

Cells grown on ferulate were used for the examination of the demethylation and fission of the aromatic ring. Cultures were harvested before exhaustion of the substrate because the amount of induced enzyme would be expected to fall in the absence of specific inducer.

Fig. 1 shows the oxidation of vanillate, protocatechuate and β -oxoadipate in phosphate and tris buffers, by the combined soluble (S_1) and particulate (P_1) fractions left after freeing press-disrupted *Pseudomonas* T of whole cells by centrifugation (18000g for 15min.). The lower rates of oxygen uptake in phosphate buffer are compatible with its known inhibitory effect on terminal oxidation. The significance of the oxygen uptake is discussed below.

The attack on vanillate was evidently enzymic since: (a) no oxygen was absorbed by incubated

mixtures of vanillate and buffer at pH 7.8; (b) no oxygen was absorbed by identical mixtures in the presence of cell-free extract previously heated at 100° for 5min.; (c) the rate of vanillate oxidation was proportional to the protein concentration, albeit only above a minimum protein concentration of approx. 15mg./ml. (Fig. 2) (a possible explanation of this effect is given below); (d) activity was completely removed after a short dialysis, whereas undialysed extract kept for a similar period was only partially deactivated, which suggested an enzyme-cofactor involvement.

Activity of supplemented bacterial extracts. In addition to the effect of dialysis, the deactivation by dilution suggested cofactor participation; in this case loss of activity could be due either to a decrease in the concentration of endogenous cofactor to an ineffective level or possibly to oxidative inactivation in the resultant lowered thiol concentration.

The effects of supplementation on inactive dilute S_1 extracts with cysteine, homocysteine, thioglycollate, 2,3-dimercaptopropan-1-ol, GSH, NAD^+ , $NADP^+$, $NADH$, $NADPH$, ATP, ADP, tetrahydrofolate and metal ions singly or in selected mixtures were investigated. $NADPH$ stimulated activity considerably; $NADP^+$ was not so effective

Fig. 2. Rates of vanillate oxidation at various concentrations of unsupplemented cell-extract from *P. fluorescens* T. Warburg conditions were as given in Fig. 1. Each flask contained crude cell-free extract of *P. fluorescens* T (the final concentration of soluble protein fraction only is indicated on the graph) and 0.067M-phosphate buffer, pH 7.8, to 1.8ml. in the main well; 0.2ml. of 20% (w/v) KOH was in the centre well. Vanillate (2.0μmoles) was tipped from the side arm to initiate the reaction. Controls contained no substrate. Endogenous respiration was subtracted.

Table 1. Specificity of the cofactor requirements of *P. fluorescens* T vanillate demethylase

The incubation temperature was 30°, the total fluid volume 3.0ml. and the gas phase air. Warburg flasks contained fraction S_1 equivalent to 20.0mg. of protein (the soluble fraction of disrupted *P. fluorescens* T cells after centrifugation at 72000g for 45min.), 1μmole of thiol, 1μmole of nucleotide, 2.8μmoles of *p*-chloromercuribenzoate as indicated, and 0.067M-phosphate buffer, pH 7.8, to 2.8ml. in the main well; 0.2ml. of 20% (w/v) KOH was in the centre well. The reaction was initiated by tipping 3.0μmoles of vanillate from the side arm. Controls contained no substrate.

Supplementation	Specific activity (μl. of O ₂ taken up/hr./mg. of protein)	Final O ₂ uptake (moles/mole of vanillate)
GSH + NADP ⁺	24.0	1.92
GSH + NAD ⁺	21.6	1.85
Cysteine + NADP ⁺	10.5	1.39
Cysteine + NAD ⁺	8.4	1.03
2,3-Dimercapto- propan-1-ol + NADPH	0.0	—
Thioglycollate + NADP ⁺	20.5	1.60
Homocysteine + NADP ⁺	8.5	1.42
GSH + NADP ⁺ + <i>p</i> - chloromercuribenzoate	0.0	—

but was active in the presence of GSH. NAD⁺ did not elicit quite such a high level of stimulation as NADP⁺, and of the thiols tested GSH evoked the best activity (Table 1).

In an extract S₁+P₁ insufficiently diluted to remove activity (22mg. of protein/ml.), NADPH-GSH mixtures gave a 3½-fold stimulation as judged by initial rates of oxygen uptake (Fig. 3). Supplementation was effective with extracts further diluted to contain 5.0mg. of protein/ml.

The behaviour of such supplemented extracts was characterized by an initial rapid uptake of 2moles of oxygen/mole of vanillate, followed by a slower uptake of 0.5mole of oxygen/mole, with accumulation of material positive to Rothera's (1908) test for ketonic compounds. Concentrated unsupplemented extracts gave a rapid uptake of 2.5moles of oxygen/mole, followed by a slow rise towards complete oxidation, after which no Rothera-positive material was detectable. An explanatory scheme of the pattern of these curves is given below but absorption of 2.5moles of

oxygen/mole accounts for oxidative demethylation followed by ring-cleavage to β -oxoadipate. Cessation of oxygen uptake beyond 2.5moles/mole in the presence of more-dilute cell extract is explicable on the grounds of inadequate 'sparker' concentration of succinyl-CoA required for the metabolism of β -oxoadipate (Kilby, 1951). Oxygen uptakes with protocatechuate (1mole of oxygen/mole), likewise with accumulation of Rothera-positive material, and β -oxoadipate (nil) under the same conditions tended to confirm this view. With concentrated extracts Rothera-positive material could only be detected from vanillate and protocatechuate metabolism when oxidation was terminated at 2.5 and 1moles of oxygen/mole respectively.

The removal of the particulate fraction (P₁) of the cell-free system by centrifugation as described

Fig. 3. Restimulation of *P. fluorescens* T vanillate-demethylase activity by additions of GSH, NADP⁺ and NADPH to diluted undialysed cell extracts. Warburg conditions were as given in Fig. 1. Double- or single-side-arm flasks contained crude cell extract of *P. fluorescens* T containing 40mg. of soluble protein, NADP⁺, NADPH and GSH as indicated, and 0.067M-phosphate buffer, pH 7.8, to 1.8ml. in the main well; 0.2ml. of 20% (w/v) KOH was in the centre well. Vanillate (1.0 μ mole), tipped from the side arm, initiated the reaction. GSH was tipped from the second side arm where indicated. Controls contained no substrate. Endogenous respiration was subtracted. Measurements were made in the presence of: Δ , no additions; $\bullet$, 1.0 μ mole of NADPH and 1.0 μ mole of GSH. In a separate experiment, where the concentration of soluble protein in the crude cell crush was 31mg., measurements were made in the presence of: $\circ$, 1.0 μ mole of NADP⁺, with 1.0 μ mole of GSH tipped at 20 min.

Fig. 4. Oxidation of vanillate, protocatechuate, formaldehyde and formate in the presence of *P. fluorescens* T cell-free fractions S₁ and P₁. Warburg conditions were as given in Table 1. Double- or single-side-arm flasks contained fractions S₁ (15mg. of protein) and/or P₁ (8.2mg.) (the soluble and particulate fractions respectively derived by centrifugation of crude cell extract of *P. fluorescens* T at 72000g for 45 min.), 1.0 μ mole of NAD⁺, 1.0 μ mole of NADP⁺, 2.0 μ moles of GSH and 0.067M-phosphate buffer, pH 7.8, to 2.8ml. in the main well; 0.2ml. of 20% (w/v) KOH was in the centre well. Substrate (3.0 μ moles), tipped from the side arm, initiated the reaction. Where indicated, fraction P₁ was tipped from the second side arm. Controls contained no substrate. Endogenous respiration was subtracted. $\bullet$ — $\bullet$, Vanillate with S₁+P₁; $\bullet$ — $\bullet$, vanillate with S₁, P₁ tipped from second side arm; $\bullet$ — $\bullet$, formaldehyde with S₁, P₁ tipped from second side arm; $\circ$ — $\circ$, formate with P₁; Δ — Δ , protocatechuate with S₁+P₁.

in the Experimental section had no effect on the initial rate of vanillate oxidation by soluble fraction S_1 , but resulted in the oxidation being terminated at 2 moles of oxygen/mole. Oxidation of protocatechuate was unaffected. Readdition of the washed particulate fraction suspended in buffer restored the slow uptake of 0.5 mole of oxygen/mole of vanillate. It is shown below that this particulate fraction catalysed the oxidation of formate derived from the methoxyl side chain of vanillate. The demethylating activity therefore resided entirely in the soluble fraction of the extract. For this reason the particulate fraction was generally removed from the soluble fraction in subsequent experiments (Fig. 4).

The *O*-demethylase catalysed the oxidation optimally at approx. pH 7.8 (Fig. 5), and at between 25° and 30°. Maximal stimulation of oxidation occurred with 0.36 μ mole of NADPH/ml. and between 0.36 and 0.71 μ mole of GSH/ml. in the presence of a protein concentration of 7 mg./ml. Higher concentrations of nucleotide and thiol

evoked no further stimulation and NADPH at 3.6 μ moles/ml. was slightly inhibitory.

Activity removed by 12 hr. dialysis of fraction S_1 at 4° against 0.9% sodium chloride (100 vol.) was not restored by supplementation with NADPH and GSH, which indicated either further cofactor requirement or that the enzyme was destroyed in the process. Additional supplementation with the other cofactors named above did not restore activity. EDTA (1 mM) and 8-hydroxyquinoline (1 mM) totally inhibited activity of undialysed extract, but several metal ions, including Mg^{2+} , Fe^{2+} , Co^{2+} and Mn^{2+} , did not reverse the inhibition or restore the activity of dialysed extract.

Cyanide inhibited vanillate oxidation totally at 10 mM and by 20% at 1 mM. The need to use such high concentrations to effect any inhibition suggested that cytochrome did not participate in the reaction. Attempts were therefore made to separate the cytochrome complex from the demethylase system by prolonged centrifugation. Other methods of resolution would have involved dilution of the extract or loss of the unknown cofactor(s) during dialysis. Centrifugation of the red-orange S_1 extract (35 mg. of protein/ml.) at 161 000 *g* for 1½–3 hr. yielded a red pellet of considerable size. The resultant supernatant, pale yellow after centrifugation for 3 hr., was almost as active towards vanillate as the original extract. The red pellet, which commonly contained about 37% of the total protein after centrifugation of fraction S_1 for 1½ hr., and about 66% after 3 hr., produced absorption bands at 527 and 556 $m\mu$ in a Hartridge reversion spectroscope. Centrifugation for 6 hr. produced a band of colourless supernatant material, S_2 (6 hr.), at the top of the cup, which was inactive towards both vanillate and protocatechuate. The resuspended pellet, P_2 (6 hr.), contained all the activity towards protocatechuate, and now accounted for some 76% of the total protein present. Although by itself inactive towards vanillate, fraction P_2 (6 hr.) restored oxidizing activity when recombined immediately with fraction S_2 (6 hr.), but not after 24 hr. of separate storage of the fractions at -20°. These results suggested that the demethylase complex did not require cytochrome participation, since most of the coloured material could be removed without affecting activity significantly. It is, however, likely that cytochrome removal was not complete, since oxygen uptake with vanillate was greater than that corresponding to accumulation of formaldehyde, which was the first product detectable in the oxidation of the side chain, and the further metabolism of which by crude cell extracts was shown to require cytochrome. Therefore definite exclusion of cytochrome participation is not demonstrated by these results, nor do they show conclusively

Fig. 5. Effect of pH on the rate of vanillate oxidation by *P. fluorescens* T vanillate demethylase. Warburg conditions were as given in Table 1. In phosphate buffer (●) each flask contained: fraction S_1 (soluble fraction derived from *P. fluorescens* T cell crush by centrifugation at 72 000 *g* for 45 min.) equivalent to 17.5 mg. of protein, 2.0 μ moles of GSH, 1.0 μ mole of NADPH, and 0.067 M-phosphate buffer to 2.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. Vanillate (2.0 μ moles), tipped from the side arm, initiated the reaction. In tris buffer (○) each flask contained 2.0 μ moles of vanillate, 2.0 μ moles of GSH, 1.0 μ mole of NADPH and 0.1 M-tris buffer to 2.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. Fraction S_1 (see above; 14 mg. of protein), tipped from the side arm, initiated the reaction. Controls contained no substrate.

that cytochrome was required for demethylase action, as suggested by loss of demethylating activity from the supernatant concomitantly with sedimentation of the last traces of colour. As the protocatechuate-oxidizing activity was also sedimented at the same time, and as this enzyme was later identified with the soluble protocatechuate 3,4-oxygenase described by Stanier & Ingraham (1954), it is equally likely that removal of demethylating activity from a soluble fraction by centrifugation for 6 hr. was due to sedimentation of the demethylase itself. The fraction S_2 (6 hr.) (protein content usually about 7 mg./ml.) therefore probably contained the cofactor(s) necessary for the vanillate demethylase sedimented in the pellet P_2 (6 hr.). Attempts to detect and identify the active factors in the supernatant by u.v. and visible spectroscopy and chromatography failed. The loss of activity that occurred when the fractions P_2 and S_2 were separately stored for 24 hr. may have been due to oxidation of an essential factor present in the soluble fraction. Experiments in which the soluble fraction resulting from a 3 hr. centrifugation, S_2 (3 hr.), and the resuspended particles, P_2 (3 hr.), were stored separately both in air and under nitrogen showed that the soluble fraction stored under nitrogen was almost as active towards vanillate 24 hr. after preparation as it was initially. The activity of material was halved after storage under air. Immediate recombination of the air-stored soluble material with either the air- or nitrogen-stored particulate material (inactive alone towards vanillate), on dilution in buffer when carrying out assay, restored activity almost completely. This restimulation was too great to be due to sedimented vanillate demethylase (Table 2). Stimulation by the particulate fraction may have been due to reactivation of a labile cofactor, e.g. reduction of a substance that was inactive in the

oxidized state by a particulate component (mitochondria?), or some other process that required energy.

A factor, apparently oxygen-labile, could also be prepared from cell extract by a modified dialysis procedure. Extract was dialysed against an equal volume of buffer in the presence of 10 mM-GSH for 12 hr. in a briskly agitated Universal bottle. Air was excluded from the system by displacement with nitrogen. The diffusate partially restored the demethylating activity of extract that had been completely inactivated by dialysis against 100 vol. of buffer for 24 hr. (Table 3). The protein fraction was still partially active after dialysis by the modified procedure and was stimulated by the readdition of diffusible supplement, although full activity was never regained. The procedure repeated in the presence of air and absence of thiol yielded a diffusate that was less active than the preparation made in the presence of thiol under nitrogen, or not active at all. The protein fraction after dialysis in absence of thiol always possessed a higher specific activity than extract dialysed in the presence of 10 mM-GSH. Greatest specific activity in reconstructed systems was therefore obtained with the combination of extract dialysed in the absence of thiol and presence of air with diffusate prepared in 10 mM-GSH and under nitrogen. The activity of diffusates was not conserved by storage under nitrogen in a Thunberg tube beyond 6 hr. of opening the bottle in which they were prepared. The activity of diffusible supplement varied with the batch of extract from which they were prepared. Alternative ways of extracting supplement (e.g. chilled ethanol and acetone cell extracts) failed. The diffusible supplement did not restore the activity of ammonium sulphate precipitates from active cell crushes.

The undefined cofactor(s) required by vanillate

Table 2. *Effect of oxygen on the activity of the soluble fraction from disrupted P. fluorescens T cells centrifuged at 161 000 g for 3 hr.*

Warburg conditions were as given in Table 1. Each Warburg flask contained soluble yellow fraction S_2 (derived by centrifugation of fraction S_1 at 161 000 g for 3 hr.) equivalent to 4.9 mg. of protein and/or the particulate fraction P_2 (derived by the same procedure), 10.1 mg. of protein, 1 μ mole of NADPH, 2 μ moles of GSH and 0.067 M-phosphate buffer, pH 7.8, to 2.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. Vanillate (3 μ moles), tipped from the side arm, initiated the reaction. Controls contained no substrate.

Fraction stored under	Rate of O_2 uptake (μ l./min.)		Rate at 24 hr. Rate at 0 hr. $\times 100$
	At 0 hr.	At 24 hr.	
Air			
S_2	5.7	2.6	45.6
S_2		5.5	96.5
P_2 + S_2		6.5	97.0
S_2 + P_2		6.0	89.5
$S_2 + P_2$		6.0	89.5
$S_2 + P_2$	6.7	6.5	97.0
$S_2 + P_2$			

Table 3. *Oxygen-lability of a diffusible supplement prepared from the soluble (S₁) fraction from disrupted P. fluorescens T cells*

From the same sample of material two portions of the S₁ extract of *P. fluorescens* T cells were dialysed for 12 hr. at 4° against equal volumes of buffer, one in the presence of 10 mM-GSH and under N₂, the other in air and without added GSH. The buffer outside the dialysis bag at the end of the 12 hr. period contained the diffusible supplement. A further sample of the same material was held at 4° for 12 hr., without dialysis, and represented the control. Extract was also dialysed exhaustively against 100 vol. of buffer for 24 hr. at 4°. Warburg conditions were as given in Table 1. Flasks contained variously extract dialysed under air (20.4 mg. of protein) ± diffusible supplement (equivalent volume), extract dialysed under N₂ (28.0 mg. of protein) ± diffusible supplement (equivalent volume), undialysed extract (20.0 mg. of protein), or exhaustively dialysed extract (17.5 mg. of protein), GSH to a final value of 10 μmoles, 1.0 μmole of NADPH and 0.067 M-phosphate buffer, pH 7.8, to 2.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. Vanillate (2 μmoles), tipped from the side arm, initiated the reaction. Controls contained no substrate.

Conditions of dialysis		Specific activity towards vanillate (μl. of O ₂ uptake/hr./mg. of protein)
Protein fraction prepared under	Diffusible supplement prepared under	
Air	—	3.08
Air	Air	3.83
Air	N ₂	5.74
N ₂	—	1.18
N ₂	Air	1.39
N ₂	N ₂	1.82
Control		12.2
Exhaustively dialysed under air	—	0.00
Exhaustively dialysed under air	N ₂	4.8

demethylase in addition to NADPH and GSH was apparently similar to that required by a cell-free demethylating preparation, specific for *p*-methoxybenzoate, from another soil pseudomonad currently under investigation in this Department. Extract specifically active towards *p*-methoxybenzoate restored the ability of dialysed extracts of *P. fluorescens* T to oxidize vanillate. Similarly a preparation of *P. fluorescens* T that did not attack *p*-methoxybenzoate restored the ability of dialysed extracts of the other organism to oxidize *p*-methoxybenzoate.

Specificity of the demethylating enzyme. Several methoxybenzoate derivatives and an ethoxybenzoate were tested with cell-extract preparations. The results indicated that the demethylating enzyme was specific for a methoxyl group *meta* to

Table 4. *Substrate specificity of P. fluorescens T vanillate demethylase*

Warburg conditions were as given in Table 1. Each flask contained 18.5 mg. of protein of fraction S₁ (the soluble fraction from ruptured *P. fluorescens* T cells after centrifugation at 72000g for 45 min.), 2 μmoles of GSH, 1 μmole of NADPH and 0.067 M-phosphate buffer, pH 7.8, to 2.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. The reaction was initiated by tipping 2 μmoles of substrate from the side arm. The control contained no substrate.

Substrate	Rate of oxidation (% of that with vanillate)
<i>o</i> -Methoxybenzoate	10
<i>m</i> -Methoxybenzoate	63
<i>p</i> -Methoxybenzoate	0
Veratrate	63
Guaiacol	5.3
<i>o</i> -Vanillate	5.3
Isovanillate	0
3-Ethoxy-4-hydroxybenzoate	0
Piperonylate	0

a carboxyl group (Table 4). Both these groups were necessary for activity: 3-ethoxy-4-hydroxybenzoate was not metabolized, and guaiacol, lacking the carboxyl group, was not attacked. The hydroxyl group was not necessary for activity: the rates of oxidation of *m*-methoxybenzoate and veratrate (50% that for vanillate) are compatible with primary oxidation proceeding to *m*-hydroxybenzoate and isovanillate respectively (1 mole of oxygen/mole), without further uptake of oxygen necessary for the faster ring-fission process. These two metabolites were shown to accumulate (see below). *o*-Vanillate was not attacked, perhaps because a hydroxyl group *ortho* to both the carboxyl and methoxyl groups excluded attachment of the substrate to the enzyme. It is also noteworthy that piperonylate (3,4-methylenedioxybenzoate) was not oxidized by vanillate demethylase.

Oxidative metabolism of the methoxyl group

Different batches of the soluble fraction (S₁) of *P. fluorescens* T extract oxidatively attacked formaldehyde with uptake of 0.35–0.5 mole of oxygen/mole (Fig. 4). In the presence of the particulate fraction (P₁) formate was also attacked with absorption of nearly 0.5 mole of oxygen/mole. The combined soluble and particulate fractions oxidized formaldehyde with uptake of 0.85–1.0 mole of oxygen/mole. Methanol was not attacked. The formate- and formaldehyde-oxidizing activities were lower in gluconate- and butyrate-grown cells than in cells grown on ferulate.

The reaction sequence envisaged for methoxyl group removal was therefore:

the first two steps being carried out by enzymes in the soluble fraction, and the third by the particulate fraction.

Metabolism of the methoxyl group to formaldehyde. Free formaldehyde was not detected during or at the termination of vanillate metabolism, but, in the presence of freshly prepared neutral semi-carbazide (18mM) and without GSH supplementation, oxygen uptake with vanillate in the presence of soluble fraction was halted at nearly 1.5 moles of oxygen/mole, and formaldehyde oxidation did not proceed. Addition of further thiol was avoided since it also stimulated the enzyme catalysing formaldehyde oxidation (see below). Vanillate oxidation, normally incomplete without supplementary GSH, was made quantitative by supplying NADPH in amounts equimolar with the substrate concentrations. Protocatechuate oxidation was not affected by the presence of the trapping reagent. Chromotropic acid estimations made on these partially deproteinized incubation mixtures indicated an accumulation of 0.88 mole of formaldehyde/mole of vanillate, and none from protocatechuate. Formaldehyde recovered from formaldehyde incubations amounted to 0.90 mole/mole supplied.

Further qualitative proof of the oxidation of the methoxyl group to formaldehyde was obtained in the following manner. Formaldehyde reversibly combines with homocysteine to yield the heterocyclic ring-compound thiazine-4-carboxylic acid (Ratner & Clarke, 1937; Strittmatter & Ball, 1955). Homocysteine did not act as a cofactor for either the vanillate- or formaldehyde-oxidizing enzyme; therefore incubation mixtures were set up containing substrate, nucleotide and cell extract, with homocysteine as trapping agent. It was necessary to supply in addition some GSH to ensure that the oxidation of vanillate proceeded to a value of at least 1.5 moles of oxygen/mole. Mixtures of 5 μ moles of substrate, 5 μ moles of homocysteine and 1 μ mole of GSH showed no oxygen uptake with

formaldehyde as substrate, but vanillate oxidation could not always be arrested at a level corresponding exactly with formaldehyde accumulation. The reason for this discrepancy is not known. With homocysteine present, thiazine-4-carboxylic acid could regularly be extracted when formaldehyde or vanillate, but not protocatechuate, was substrate.

Metabolism of the methoxyl group to formate. With soluble fraction S₁, in the absence of semi-carbazide as trapping agent, oxygen uptake with vanillate approached 2 moles of oxygen/mole. The course of vanillate, formaldehyde and protocatechuate oxidation was followed in the presence of GSH, NADP⁺ and NAD⁺. NAD⁺ was incorporated to ensure complete metabolism of formaldehyde (see below). Formate-extract mixtures were included as controls. At the termination of oxidation, flask contents were acidified with 0.1 vol. of 2N-sulphuric acid and distilled from the frozen state to dryness *in vacuo* by using the micro-distillation technique described by Grant (1946). Samples of the distillate were estimated enzymically for formate content. Nearly quantitative yields of formate were recovered from vanillate (0.97 mole/mole), formaldehyde (0.82 mole/mole) and formate (1.00 mole/mole) incubations, but none was obtained from protocatechuate or controls without substrate.

Qualitative identification of formate was also provided by chromatography. Ammoniacal silver nitrate and bromocresol green sprays demonstrated the accumulation in vanillate incubations of material running identically with, and having the same reactions as, authentic formate. Formate was also recovered from formate incubations, but not from protocatechuate or from control incubations containing no substrate.

In the presence of combined soluble and particulate fractions no formate was detected at the termination of oxidation.

Metabolism of formaldehyde to formate. The enzyme complex catalysing the oxidation of formaldehyde to formate was resolved into two parts:

(1) An enzyme resembling the formaldehyde dehydrogenase described by Strittmatter & Ball

Scheme 1.

(1955), which oxidized formaldehyde by removal of 2H to NADP⁺ and replaced them with water. GSH was also a cofactor: the substrate for oxidation was therefore presumed to be the condensation product between formaldehyde and GSH, namely *S*-hydroxymethylglutathione.

(2) A factor, probably cytochrome, which catalysed the back-oxidation of NADH to NAD⁺ with uptake of approx. 0.5 mole of oxygen/mole of formaldehyde. The sequence of reactions appeared to consist of those given in Scheme 1.

Soluble fraction (*S*₁) catalysed the uptake of 0.5 mole of oxygen/mole of formaldehyde and its activity was abolished by dialysis against 0.9% sodium chloride for 12 hr. Activity could be restored by additions of GSH and NAD⁺ together, but not separately (Table 5).

Spectrophotometric measurements of the progress of the reaction made at 340 mμ (*E*_{max.} for NADH) indicated that 340 mμ-absorbing compound was formed only in the presence of all four components, namely GSH, NAD⁺, formaldehyde and cell extract (Fig. 6). With boiled extract no accumulation was observed. The results suggested that a rapid production of NADH ensued on the addition of NAD⁺, the reaction mixture giving a peak of maximum absorption after about 5 min. under the test conditions, and then the absorption slowly declined back to the original value. This cycle could be repeated several times by sequential additions of formaldehyde, at an accelerated rate if aeration was applied, or halted at the NADH level if a barrier to aeration was provided by layering the cuvette contents with octan-2-ol.

Table 5. Cofactor requirements of the formaldehyde-oxidizing activity in *P. fluorescens* T

Warburg conditions were as given in Table 1. Each flask contained fraction *S*₁ equivalent to 17.6 mg. of protein (supernatant from disrupted *P. fluorescens* T cells centrifuged at 72000*g* for 45 min.), 1 μmole of NAD⁺, 2 μmoles of GSH as indicated and 0.067 M-phosphate buffer, pH 7.8, to 2.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. Formaldehyde (3 μmoles), tipped from the side arm, initiated the reaction. Controls contained no formaldehyde. Dialysis of fraction *S*₁ was carried out against two changes of 100 vol. of 0.9% NaCl for 24 hr.

Incubation mixture	Specific activity (μl. of O ₂ taken up/hr./mg. of protein)
Undialysed <i>S</i> ₁ + GSH + NAD ⁺	6.82
Dialysed <i>S</i> ₁	0.34
Dialysed <i>S</i> ₁ + GSH	0.51
Dialysed <i>S</i> ₁ + NAD ⁺	0.34
Dialysed <i>S</i> ₁ + GSH + NAD ⁺	5.11

The NAD⁺-reducing activity was separated from the NADH-oxidizing activity by ammonium sulphate fractionation. Table 6 shows that the NAD⁺-reducing activity precipitated between 0.4 and 0.5 saturation, and that it was identical in its cofactor requirements with animal formaldehyde dehydrogenase (Strittmatter & Ball, 1955). All the NADH-oxidizing activity was precipitated with the electron-transport system between 0 and 0.4 saturation. Further attempts to purify the NAD⁺-reducing fraction by reprecipitation with ammonium sulphate resulted in complete loss of activity.

The partially resolved NAD⁺-reducing fraction, which is tentatively named bacterial formaldehyde dehydrogenase, was more active at pH 8.0 than pH 6.8, and lost activity rapidly after fractionation; storage for 24 hr. at -20° lowered the activity to 42% of the original value. Enzyme activity was destroyed by boiling. The rate of NADH production was decreased when the formaldehyde concentration was raised above 0.8 μmole/ml. (other

Fig. 6. Activity of *P. fluorescens* T formaldehyde dehydrogenase: NAD⁺ reduction and back-oxidation by crude *S*₁ extract in the presence of formaldehyde at 22°. Cuvettes contained *P. fluorescens* T cell-extract fraction *S*₁ equivalent to 8.6 mg. of protein (the soluble fraction of the cell extract derived by centrifugation at 72000*g* for 45 min.), 1.0 μmole of formaldehyde, 1.0 μmole of GSH and 0.067 M-phosphate buffer, pH 7.8, to a final volume of 2.8 ml. The addition of 1.0 μmole of NAD⁺ initiated the reaction and extinction values were measured at 340 mμ. Further formaldehyde (1.0 μmole) was added as indicated. Controls contained no NAD⁺. ●, Formaldehyde + GSH + NAD⁺; Δ, with the omission of any one of the following: formaldehyde, GSH, extract or NAD⁺, the last being read against a control of buffered extract alone; ○, formaldehyde + GSH + NAD⁺, with the cuvette contents layered with octan-2-ol.

Table 6. *Fractionation of the soluble fraction (S₁) from P. fluorescens T by ammonium sulphate to separate formaldehyde dehydrogenase from the NADH-oxidizing system*

Fraction S₁ was the supernatant from a cell-free extract centrifuged at 72000g for 45 min. Spectrophotometric measurements were made at 340m μ (at 22°) and the total volume in each cuvette was 2.5 ml. Cuvettes contained 0.005–0.05 ml. of enzyme extract, 2.0 ml. of de-aerated 0.067 M-phosphate buffer, pH 7.8, 2 μ moles of GSH and 2 μ moles of formaldehyde. NAD⁺ (0.5 μ mole) was added to initiate the reaction. Controls contained no NAD⁺. A layer of octan-2-ol was placed over preparations containing cytochrome to prevent back-oxidation of NADH.

Fraction	Degree of (NH ₄) ₂ SO ₄ saturation required for precipitation	Protein recovered (mg.)	Specific activity (ΔE /min./mg. of protein)	Recovery (%)	Purification
NP ₁	0.40	393.0	Negligible	—	—
NP ₂	0.40–0.50	72.5	0.225	13.65	1.09
NP ₃	0.50–1.00	113.0	Negligible	—	—
S ₁	—	578.5	0.207	100	1.00

Table 7. *Formate production from formaldehyde in the presence of partially resolved P. fluorescens T formaldehyde dehydrogenase*

Incubation time was 3 hr. at 22°. Complete incubation mixtures consisted of substrate formaldehyde as indicated, 3 μ moles of GSH, NAD⁺ as indicated, *P. fluorescens* T formaldehyde dehydrogenase (fraction NP₂; see Table 6) equivalent to 0.78 mg. of protein and 0.067 M-phosphate buffer, pH 7.8, to 2.5 ml. Incubation mixtures were varied as follows: tube 1, no formaldehyde, 3.0 μ moles of NAD⁺; tube 2, 6.0 μ moles of formaldehyde, 7.0 μ moles of NAD⁺; tube 3, 6.0 μ moles of formaldehyde, 3.0 μ moles of NAD⁺; tube 4, 3.0 μ moles of formaldehyde, 3.5 μ moles of NAD⁺; tube 5, 3.0 μ moles of formaldehyde, no NAD⁺; tube 6, 3.0 μ moles of formaldehyde, 3.5 μ moles of NAD⁺ and no GSH; tube 7, 3.0 μ moles of formaldehyde, 3.5 μ moles of NAD⁺ with fraction NP₂ heat-inactivated (100° for 10 min.); tube 8, 3.0 μ moles of formaldehyde, 3.5 μ moles of NAD⁺ and no fraction NP₂.

Mixture no.	Formate expected (μ moles)	Formate found (μ moles)	Formaldehyde expected (μ moles)	Formaldehyde found (μ moles)	Theoretical total (μ moles)	Actual total (μ moles)
1	—	—	—	—	—	—
2	6.0	5.05	0.0	0.31	6.0	5.36
3	3.0	2.30	3.0	0.42	6.0	2.72
4	3.0	2.61	0.0	0.45	3.0	3.04
5	0.0	0.15	3.0	2.04	3.0	2.19
6	0.0	0.76	3.0	3.08	3.0	2.84
7	0.0	0.0	3.0	2.52	3.0	2.52
8	0.0	0.0	3.0	2.52	3.0	2.52

reactants: NAD⁺, 0.2 μ mole/ml.; GSH, 0.4 μ mole/ml.; 0.6 mg. of protein/ml.). GSH concentrations in excess of the above value had little stimulatory effect on the rate and NAD⁺ could not be replaced by NADP⁺.

The enzyme catalysed the formation of approx. 0.8 mole of NADH for every mole of NAD⁺ and formaldehyde supplied. Estimation of the formate produced showed that, for every mole of formaldehyde, an average value of 0.82 mole of formate accumulated (Table 7).

Metabolism of formate to carbon dioxide. The washed particulate material (P₁) resulting from cell crushes of *P. fluorescens* T catalysed the oxidation of formate with uptake of 0.5 mole of oxygen/mole. Dialysis over a long period did not decrease the activity, which spontaneously began to decline about 2 days after extraction.

The optimum pH for activity was about 6.5, and the system was strongly inhibited by 1 mM-cyanide. The enzyme complex was therefore apparently similar to the formate dehydrogenase–cytochrome b₁ system in *Escherichia coli* described by Gale (1939), and to a similar system in *Pseudomonas* reported more recently by Dagley & Gibson (1965), but attempts to detect cytochrome reduction in the presence of formate, with a Hartridge reversion spectroscope, proved unsuccessful, probably because the particulate material from this strain of *Pseudomonas* was excessively opaque and showed considerable light-scattering.

Ring metabolism

Metabolism of vanillate to protocatechuate. No convincing direct evidence has been obtained that

vanillate ring metabolism proceeds via protocatechuate, but this is almost certainly the pathway because:

(1) Protocatechuate was the only *o*-dihydroxy compound isolated by lead acetate precipitations (Evans, 1947) of supernatants from vanillate-grown cultures. Trace amounts were occasionally detected by chromatography (Whatman no. 542 paper, solvent C, spray I.C.I. 5091).

(2) Although catechol was attacked by very concentrated unsupplemented cell extracts with uptake of 1 mole of oxygen/mole, the dilute supplemented extracts that attacked vanillate and protocatechuate oxidized catechol very slowly or not at all.

(3) Cells adapted to vanillate were also fully adapted to protocatechuate but only partially to catechol. Cells not adapted to vanillate were not adapted to protocatechuate.

(4) The oxidation of *m*-methoxybenzoate and veratrate by these cell extracts led to the accumulation of *m*-hydroxybenzoate and isovanillate respectively, which were identified by paper chromatography. No evidence of further oxidation of these products by whole cells or extracts was obtained.

The course of oxidation of 2 μ moles of each substrate was followed manometrically. On completed oxidation, flask contents were deproteinized with 0.1 vol. of 10% trichloroacetic acid, centrifuged and continuously extracted with ether for 6 hr. Chromatography of the ether-soluble substances on Whatman no. 542 paper with solvent C overnight revealed the accumulation of compounds having the same R_f , colour reaction with I.C.I. 5091 spray reagent and appearance under u.v. light as *m*-hydroxybenzoate (from *m*-methoxybenzoate) and isovanillate (from veratrate) in quantitative amounts.

Protocatechuate was never observed to accumulate during vanillate oxidation by cell-free extracts. Attempts to halt the oxidation of protocatechuate failed, because none of the potential non-competitive inhibitors tested would act selectively on protocatechuate oxygenase, and the competitive inhibitors proved ineffective when tested at the cell-extract concentrations required for the metabolism of vanillate. The very rapid rate of protocatechuate oxidation by extracts would make its accumulation unlikely in the absence of inhibitors. Our failure to separate the vanillate-oxidizing system from that attacking protocatechuate also precluded a demonstration of protocatechuate accumulation.

Metabolism of protocatechuate to β -oxoadipate. Vanillate and protocatechuate metabolism by dilute supplemented extract led to the accumulation of Rothera-positive material, which also combined with 2,4-dinitrophenylhydrazine to give a deriva-

tive extractable by the method of Friedemann & Haugen (1943). A preliminary boiling of the sample before extraction yielded a compound that ran identically with laevulate 2,4-dinitrophenylhydrazine on chromatography in both solvents C and D overnight (Whatman no. 542 paper). Unfortified extract at lower dilution did not attack vanillate, but metabolized protocatechuate, with accumulation of Rothera-positive material, giving a 2,4-dinitrophenylhydrazine identical with the laevulate derivative.

The compound accumulated was therefore probably β -oxoadipate. Estimations by the catalytic decarboxylation procedure of Sistrom & Stanier (1953) on the products of vanillate metabolism in dilute supplemented extracts and protocatechuate oxidation by supplemented and unsupplemented extract showed that approx. 0.8 mole of oxo acid was formed/mole of substrate. Fortified dilute extract catalysed the quantitative production of oxo acid from both vanillate and protocatechuate, provided that the protein concentration was held at a minimal value, and GSH, which serves as a cofactor for the further metabolism of β -oxoadipate (Kilby, 1951), was omitted from the reaction mixture. NADPH was supplied at concentration equimolar with the substrate to ensure complete metabolism of vanillate.

Slight metabolism of β -oxoadipate was noticed under the conditions of the test, probably owing to the presence of trace amounts of succinyl-CoA needed to catalyse its degradation (Kilby, 1951).

Table 8 shows the amount of oxo acid obtained from metabolism of differing amounts of vanillate and protocatechuate compared with a control of β -oxoadipate.

The protocatechuate-oxidizing enzyme system was shown to be probably identical with the protocatechuate oxygenase described by Stanier & Ingraham (1954), in that dialysis did not decrease its activity and activity increased with increasing pH. Crude extracts catalysed the uptake of 1 mole of oxygen/mole of protocatechuate with the production of β -oxoadipate and evolution of carbon dioxide. By a series of four ammonium sulphate fractionations and a final treatment with calcium phosphate gel, the oxygenase was purified 9.6-fold. Partially purified oxygenase metabolized protocatechuate without the accumulation of Rothera-positive material and with no evolution of carbon dioxide, but instead material with a u.v. spectrum identical with that of authentic *cis-cis*- β -carboxymuconate was produced.

Scheme 2 summarizes the probable pathway of breakdown for vanillate in *Pseudomonas*. All enzymes responsible, with the exception of formate dehydrogenase, were in the soluble fraction of the cell extract.

Table 8. *β -Oxadipate production from vanillate and protocatechuate in the presence of the soluble fraction (S_1) from ruptured *P. fluorescens* T cells*

Warburg conditions were as given in Table 1. Each double-side-arm flask contained 1 μ mole of NADPH/ μ mole of substrate supplied, substrate as indicated and 0.067 M-phosphate buffer, pH 7.8, to 2.8 ml. in the main well; 0.2 ml. of 20% (w/v) KOH was in the centre well. Fraction S_1 of *P. fluorescens* T cells (soluble fraction after centrifugation at 72000g for 45 min.) equivalent to 16.8 mg. of protein, tipped from the side arm, initiated the reaction. Controls contained no substrate. β -Oxo acid was assayed as follows. To the main well of each flask was added 0.2 ml. of acetic acid and 1.0 ml. of 0.1 M-acetate buffer, pH 4.0. KOH was swabbed from centre wells and the reaction initiated with 0.4 ml. of 0.1 M-4-aminoantipyrin, tipped from the second side arm.

Substrate supplied (μ moles)	Yield of β -oxo- adipate (μ moles)	Yield (% of expected value)
Vanillate (2.0)	1.52	76
Protocatechuate (2.0)	1.34	67
β -Oxadipate (2.0)	1.52	76
Vanillate (3.0)	2.46	82
Protocatechuate (3.0)	2.57	85.5
β -Oxadipate (3.0)	2.42	80.5
Vanillate (5.0)	4.18	83.5
Protocatechuate (5.0)	3.95	79
β -Oxadipate (5.0)	4.42	88.5

Scheme 2.

DISCUSSION

Several simple compounds, e.g. coniferyl alcohol, ferulate, eugenol, isoeugenol etc., which occur in plants, have a common basic methoxyphenylpropane-type structure and it is generally accepted that the lignin macromolecule is formed by dehydrogenation and condensation reactions between phenylpropanoid precursor units (Brauns & Brauns, 1960). In lignin the phenyl group is predominantly substituted 4-hydroxy-3-methoxy (guaiacyl) with a proportion of 4-hydroxy-3,5-dimethoxy (syringyl) or 4-hydroxy substitution, dependent on the type of woody tissue. The

appearance of phenylpropane-type compounds, e.g. guaiacylpyruvate (Fukuzumi, 1960), in the microbial degradation products of lignin is therefore not surprising. For obvious reasons wood-rotting fungi have been extensively used in studies of the catabolism of lignin and related compounds. However, soil bacteria must also play a part in the process of decomposition after the death of plants, but comparatively little attention has been paid to this aspect. Fischer, Bizzini, Raynaud & Prévot (1956) and Sørensen (1962) demonstrated bacterial attack on isolated lignin and determined the amount decomposed under particular conditions. Lignans, the carbon skeleton of which was shown

to be derivable from two phenylpropane units, ferulate, by chemical oxidation (Cartwright & Haworth, 1944), are also associated with woody tissues, and their dissimilation by species of *Flavobacteria*, *Pseudomonas* and *Agrobacteria* has respectively been reported by Konetzka, Pelczar & Gottlieb (1952), Tabak, Chambers & Kabler (1959) and Sundman (1964a,b). It is noteworthy that the *Pseudomonas* used in our work not only degraded ferulate but also carried out the intermolecular dehydrogenation to the lignan-like dehydrodiferulate previously obtained *in vitro* by pure chemical methods.

Many substances have been isolated and identified in lignin dissimilation but the enzymology of their formation is largely unknown. Advances in this field may be limited, not only by uncertainty about the true structures of various native and isolated lignins, but also by lack of knowledge of the behaviour in enzymic processes of several types of chemical linkage and reactive groups of lignin. In addition to the methoxyl groups already present in precursors and which apparently are not involved in lignin biosynthesis, other ether linkages, e.g. benzofuran rings, are probably present in the lignin structure. We have therefore studied the attack by bacterial enzymes on ether linkages in substances related to lignin and are here concerned with catabolism of the methoxyl group.

Our results show unequivocally that formaldehyde (or if not the free aldehyde a combined form that yields it on treatment with ketonic reagents, e.g. semicarbazide in this context), formate and carbon dioxide are sequentially and quantitatively produced by the action of *P.*

fluorescens T enzymes on the methoxyl group of vanillate. The phenylmethyl ether bonds are notoriously stable and require drastic chemical means for fission, which contrast with the mild conditions of enzymic attack.

Several possible modes of loss of the methyl group are outlined in Scheme 3.

Both vanillate-adapted whole cells and cell-free extracts attacked formaldehyde and formate but not methanol, and so demethoxylation by route B-F is unlikely. Methane evolution by route G was not definitely excluded, and in some experiments with other pseudomonads adapted to the isomers of methoxybenzoate a small gas evolution was frequently noted in the initial stages of Warburg manometry on washed-cell suspensions plus primary substrate. Techniques and facilities for identifying small quantities of methane were not available. Failure to observe gas evolution with cells or extracts does not itself rule out methane production. The high rate and volume of oxygen uptake in further oxidation of intermediates could mask gas evolution. The isolation of formaldehyde is also not incompatible with methane or methanol production because it could arise by oxidation of either of these compounds.

Our results cannot distinguish between the direct production of protocatechuate (route A) or indirectly via *p*-hydroxybenzoate (routes B-C and B-D). Nevertheless, although *p*-hydroxybenzoate was attacked by concentrated cell extracts, no oxygen uptake was observed in the presence of the diluted supplemented preparations that dissimilated vanillate. A knowledge of oxygen uptake, or lack of it, in the initial stage of the dissimilation of

Scheme 3.

vanillate would have helped to clarify the point further, but we were unable to isolate or identify an active cofactor supplement (see below) from the dialysates of cell-free extracts, or to supply artificially cofactors that would promote activity towards vanillate when added to protein fractions separated from crude extracts by standard procedures. Moreover, *p*-hydroxybenzoate was not detectable in culture fluids of *P. fluorescens* T or during incubation in buffer of vanillate and washed vanillate-grown cells or crude and partially purified extracts therefrom. Work in this Laboratory has shown that other *Pseudomonas* strains adapted to *o*-, *m*- and *p*-methoxybenzoate oxidized the corresponding hydroxy acid at a rate low enough to allow its detection as an intermediate. Extracts of *P. fluorescens* T converted veratrate into isovanillate, which was not attacked, and *m*-hydroxybenzoate accumulated from *m*-methoxybenzoate incubations. Besides indicating the specificity of the demethylase, these results considered in the light of the reaction scheme tend to suggest that *P. fluorescens* T demethylates rather than demethoxylates vanillate. The oxidation of *p*-hydroxybenzoate by extracts does not mean that *p*-hydroxybenzoate is necessarily on the pathway of vanillate dissimilation, but it indicates that extracts may contain either a mixed-function oxidase (reaction D) or a hydase (reaction C) hydroxylating *p*-hydroxybenzoate to a state where ring-fission can occur. The likelihood of protocatechuate's being the compound undergoing fission was shown by: (a) its isolation on chromatograms from solvent extracts of growing culture supernatants and the resistance of isovanillate to oxidation by preparations that rapidly oxidized protocatechuate; (b) the conversion of vanillate into β -oxoadipate, a compound characteristic of the protocatechuate pathway of ring-fission.

Results obtained in attempts to define the cofactor requirements for vanillate demethylase were inconclusive. Some possible explanations of inactivation of extracts by dilution have already been given in the Results section. The restorative effect of NADPH and GSH on the activity of dilute extracts may equally indicate that they are effective in maintaining another cofactor, present in sub-optimum quantities, in a highly active (reduced?) state. A direct involvement of NADPH is not supported by its lack of stimulation of enzyme inactivated by dialysis. Experiments on the restoration of activity by readdition of the dialysate to enzyme protein dialysed under a variety of conditions indicated a requirement for a cofactor much more sensitive to oxygen or light or both than is NADPH itself. Folate derivatives exhibit such properties and are frequently involved in C_1 metabolism. Tetrahydrofolate alone or in com-

bination with ATP, ADP, NADPH, GSH, cobalamin, Mg^{2+} , Fe^{2+} , homocysteine or cysteine failed to replace the unknown active cofactor in dialysates prepared in the dark and under a nitrogen atmosphere. This finding does not rule out the involvement of a tetrahydrofolate derivative or another pteridine such as bipterin. The inhibition observed with chelating agents also suggests that the cofactor requirement might be satisfied by a metal ion functioning in a redox system, alone or in conjunction with an organic compound.

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