3. The specific radioactivity of the glycine isolated from fibrinogen of the adrenaline-treated rats was higher than in the controls throughout the experiment.

4. The specific radioactivity of the glycine isolated from mixed serum proteins of adrenaline-treated rats was very similar to that of glycine isolated from the same proteins of the saline controls at all times.

Our thanks are due to the Brazilian Research Council for the [α-^{14}C]glycine used in this work. We wish to express our thanks to Professor A. Neuberger, F.R.S., for helpful criticism during the writing of this paper.

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Oxidation of *p*-Cresol and Related Compounds by a *Pseudomonas*

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Phenolic compounds are oxidized, and hence removed from industrial effluents, by micro-organisms during percolation through sewage beds. Compared with purely chemical processes of extraction this method is cheap; and the problems of effluent disposal of an expanding coal-gas industry have led to increasing interest in the mechanisms by which phenols are biologically oxidized. The present investigations on *p*-cresol afford the first detailed study of the oxidation by bacteria of methyl groups of aromatic compounds; and the extension of these studies to oxidation of xylenols has provided information concerning the specificity of such mechanisms. From observations on 'simultaneous adaptation' (sequential induction) of whole cells, and isolation of *p*-hydroxybenzoic acid from a culture of *Pseudomonas* sp. growing at the expense of *p*-cresol, Smith, Jones & Evans (1952) have suggested that *p*-cresol is oxidized by way of *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid.

EXPERIMENTAL

Organism. The organism was isolated from liquid that had circulated through an experimental plant for the treatment of gas-works effluent in the Department of Coal Gas and Fuel Industries in this University. Dr G. M. Williamson kindly examined the organism and identified it as a species of *Pseudomonas*. It is a Gram-negative rod about $1\mu \times 2\mu$ with 2–6 polar flagella; it does not produce a pigment. From observations on its growth in various media, its biochemical activities and its behaviour towards penicillin, Terramycin and 2:4-diamino-6:7-di-*iso*propylpteridine it is closely related to, but not identical with, *Pseudomonas cruciviae* (Bergey, 1950). The organism was maintained by monthly serial subcultures on nutrient-agar slopes and cultures were grown with forced aeration at 30° in a medium adjusted with NaOH to pH 7, and containing, per litre: KH_2PO_4, 2 g.; $(NH_4)_2SO_4$, 1 g.; $MgSO_4,7H_2O$, 0·2 g.; *p*-cresol, 0·3 g. The relationship between bacterial crop and *p*-cresol concentration was approximately linear up to 0·3 g. of *p*-cresol/l., but at concentrations greater than 0·4 g./l. the compound was increasingly inhibitory to growth. When 25 ml. of liquid medium was inoculated from the slope, full growth was reached in 2 days. The whole of this culture was used as inoculum for 1 l. of fresh medium and after overnight growth the new culture was, in turn, used to inoculate 10 l. of medium; the cells were harvested after further growth for 18 hr. All buffer solutions contained KH_2PO_4 of the strength stated in the text, adjusted to pH 7 by addition of NaOH. For measurements of O_2 uptake, cells were washed twice with 0·1 M-phosphate buffer and suspended in this buffer to give a cell density of 10 mg. dry wt./ml. Cell-free extracts were prepared with the Hughes bacterial press (Hughes, 1951), without abrasive, at −14°. Each 1 g. of

crushed cells was taken up in 2 ml. of 0·1 M-phosphate buffer pH 7, and the pink, viscous extract freed from cell debris by centrifuging for 30 min. at 42 000 rev./min. in a preparative rotor of the Spinco model E ultracentrifuge.

Materials. Triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN), both of 95% purity, were obtained from L. Light and Co., Colnbrook, Bucks. The aromatic compounds used were B.D.H. laboratory reagents, or AnalaR grade where available. *cis-cis*-Muconic acid was a gift from Dr R. P. Linstead.

RESULTS

Oxidation by washed suspensions and cell-free extracts. Fig. 1 shows that intermediates in *p*-cresol oxidation suggested by Smith *et al.* (1952) were readily oxidized by whole cells; *p*-hydroxybenzyl alcohol was not tested since it is difficult to prepare and purify, and, if so obtained, rapidly resinifies (Peppiatt & Wicker, 1954). Members of the tricarboxylic acid cycle were also readily oxidized. The slower rate of oxidation of citrate may be due to its slow penetration to the interior of the cells, since citrate was oxidized by cell-free extracts rather more rapidly than succinate. Catechol and *cis-cis*-muconic acid, which are intermediates in the bacterial oxidation of phenol (Evans & Happold, 1939; Evans & Smith, 1951) were attacked only slowly by whole cells and not at all by cell-free extracts. After correction for the blank, total uptakes of O_2 (moles of O_2/mole of substrate) in the presence of extracts were approximately as follows: *p*-cresol, 3; *p*-hydroxybenzaldehyde, 2; *p*-hydroxybenzoic acid, 1·5 and protocatechuic acid, 1.

Conversion of p-*cresol into* p-*hydroxybenzaldehyde.* When cells were freeze-dried their ability to oxidize *p*-hydroxybenzaldehyde was largely abolished, although *p*-cresol was still attacked. Freeze-dried cells (100 mg.) suspended in 10 ml. of 0·1 M-phosphate buffer, pH 7, were shaken mechanically at 30° with 10 ml. of 0·01 M-*p*-cresol. For samples freed from cells by centrifuging, the increase in optical density was then measured at a wavelength of 286 mμ, where a maximum occurs in the absorption spectrum of *p*-hydroxybenzaldehyde. When there was no further increase the cells were removed and an acidified solution of 2:4-dinitrophenylhydrazine was added until precipitation was complete. After filtration, washing and recrystallization twice from ethanol, the hydrazone was dried in a desiccator over $CaCl_2$ *in vacuo*; m.p. 268–270° (decomp.), and 268–270° (decomp.) when mixed with authentic 2:4-dinitrophenylhydrazone of *p*-hydroxybenzaldehyde, m.p. 270–272° (decomp.). Fig. 2 shows the absorption curves of our sample and that of an authentic specimen of 2:4-dinitrophenylhydrazone of *p*-hydroxybenzaldehyde dissolved in ethanol. The maximum value of $E^{1\%}_{1\,cm.}$ for both specimens under these conditions was 630 at 395mμ.

In Fig. 1 it is seen that although whole cells oxidized *p*-cresol as rapidly as the compounds postulated as intermediates, its rate of oxidation by cell-free extracts, prepared with the Hughes press, was considerably smaller. This suggested that a factor involved in *p*-cresol oxidation had been removed from extracts during their preparation. After centrifuging at 42 000 rev./min., a suspension

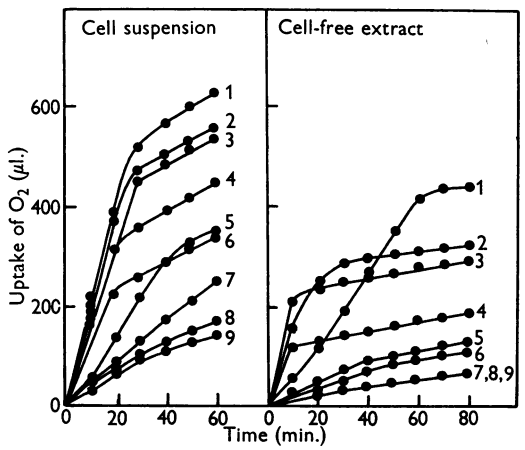

Fig. 1. Oxidation of various compounds by whole cells and cell-free extracts. Each cup contained KOH in the centre well, 1 ml. of bacterial suspension or cell-free extract and 0·1 M-phosphate buffer, pH 7, to a total liquid volume of 3 ml. Side bulbs contained 5 μmoles of the following, neutralized with NaOH where necessary: (1) *p*-cresol; (2) *p*-hydroxybenzaldehyde; (3) *p*-hydroxybenzoic acid; (4) protocatechuic acid; (5) citric acid; (6) succinic acid; (7) *cis-cis*-muconic acid; (8) catechol; (9) no substrate.

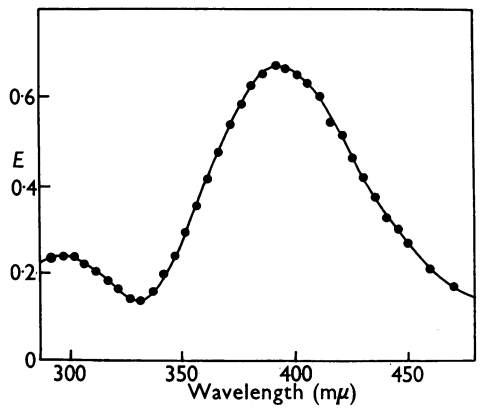

Fig. 2. Absorption spectra of two solutions of 2:4-dinitrophenylhydrazones in ethanol. The curve is for an authentic specimen of the 2:4-dinitrophenylhydrazone of *p*-hydroxybenzaldehyde and the experimental points for the 2:4-dinitrophenylhydrazone of material produced by oxidation of *p*-cresol by freeze-dried cells.

of crushed cells separated into three layers: clear-pink supernatant, a well-defined layer of pink particles in the form of a jelly and a deeper layer of whitish cell debris at the bottom of the tube. The pink-particle fraction was separated and washed by suspension in phosphate buffer followed by centrifuging and resuspension, and its effect on *p*-cresol oxidation was then investigated. From values plotted in Fig. 3, the initial rates of oxidation of *p*-cresol by clear extract, particle suspension and a mixture of the two were respectively 94, 33 and 227 μl. of O_2/30 min. Examination of the particle suspension in a Hartridge reversion spectroscope gave clear evidence of a cytochrome system; bands at 525 and 555 mμ could be seen, which disappeared on bubbling a stream of air through, and their reappearance on standing without aeration was accelerated by addition of *p*-cresol. The cell-free extracts possibly owed their pink colour to finer particles of the same nature that had resisted centrifuging. When examined in a quartz spectrophotometer, suitably diluted extracts gave absorption curves with peaks at 525 and 555 mμ and a pronounced peak in the Soret region (415 mμ). The smaller peaks were abolished when the extract was aerated between readings in the spectrophotometer.

Conversion of p-*hydroxybenzaldehyde into* p-*hydroxybenzoic acid.* Gunter (1953) published absorption curves, which we confirm, for these two compounds; *p*-hydroxybenzaldehyde shows maximum absorption at 286 mμ and *p*-hydroxybenzoic acid at 246 mμ. Their interconversion is consequently accompanied by a marked change in absorption which can be followed directly for the reaction mixture after suitable dilution, taking the reaction 'control' (i.e. enzyme with no substrate) as 'blank' in spectrophotometer readings.

After they had been precipitated with $(NH_4)_2SO_4$ and redissolved, extracts still catalysed the oxidation of protocatechuic acid readily and of *p*-cresol at a greatly reduced rate; but oxidation of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid was abolished. To each 10 ml. of crude extract was added, with stirring, 6 g. of solid $(NH_4)_2SO_4$ and the resulting precipitate centrifuged off, thoroughly drained of supernatant and redissolved in 10 ml. of 0·02 M-phosphate buffer, pH 7. From results shown in Fig. 4, an extract so treated oxidized *p*-cresol with the uptake of about 1 mole of O_2/mole of substrate; flask contents showed the absorption spectrum of *p*-hydroxybenzaldehyde and gave a

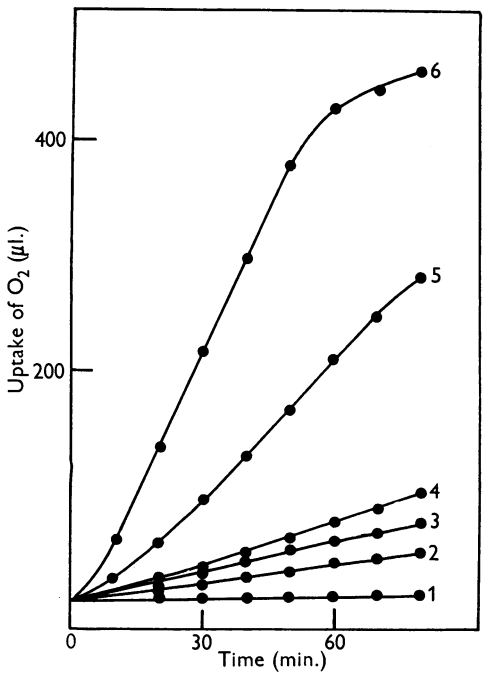

Fig. 3. Effect of a 'particle' fraction on the oxidation of *p*-cresol by a cell-free extract. Each cup contained in the centre well, 0·1 M-phosphate buffer, pH 7, to a total liquid volume of 3 ml., and (1) 0·3 ml. of 'particle' suspension; (2) 1 ml. of extract; (3) 1 ml. of extract + 0·3 ml. of 'particle' suspension; (4) 0·3 ml. of 'particle' suspension + 5 μmoles of *p*-cresol; (5) 1 ml. of extract + 5 μmoles of *p*-cresol; (6) 1 ml. of extract + 0·3 ml. of 'particle' suspension + 5 μmoles of *p*-cresol. *p*-Cresol was added from the side bulbs.

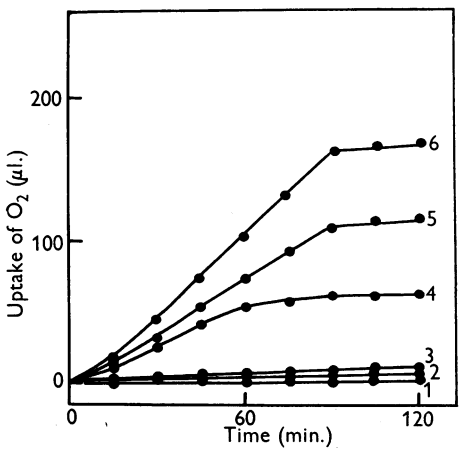

Fig. 4. Oxidation of *p*-cresol and *p*-hydroxybenzaldehyde by extracts after treatment with $(NH_4)_2SO_4$. Extract was precipitated and then redissolved in 0·02 M-phosphate buffer, pH 7. Each cup contained in the centre well 1 ml. of extract so treated, 0·1 M-phosphate buffer, pH 7, to a total liquid volume of 3 ml., KOH and (1) no additions; (2) DPN, 200 μg.; (3) *p*-hydroxybenzaldehyde, 5 μmoles; (4) *p*-hydroxybenzaldehyde, 5 μmoles + DPN, 200 μg.; (5) *p*-cresol, 5 μmoles; (6) *p*-cresol, 5 μmoles + DPN, 200 μg. *p*-Cresol and *p*-hydroxybenzaldehyde were added from the side bulbs.

precipitate with 2:4-dinitrophenylhydrazine. When DPN was present in addition to *p*-cresol the final uptake was about 1·5 mole of O_2/mole of *p*-cresol, no reaction was given with 2:4-dinitrophenylhydrazine and the spectrum corresponded to that of *p*-hydroxybenzoic acid. *p*-Hydroxybenzaldehyde was oxidized only in the presence of DPN, when 0·5 mole of O_2/mole of substrate was taken up, and at the end of the reaction the solution showed the absorption spectrum of *p*-hydroxybenzoic acid. The substrate specificities of enzymes catalysing these reactions are discussed in a later section.

Oxidation of p-*hydroxybenzoic acid and protocatechuic acid by cell-free extracts.* Uptakes of O_2 by crude extracts were compatible with the view that *p*-cresol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and protocatechuic acid were oxidized to the same end product and are members of a reaction sequence which is broken when the enzyme catalysing hydroxylation of *p*-hydroxybenzoic acid is inactivated by treatment with $(NH_4)_2SO_4$. On the assumption that this effect was due to dissociation of a cofactor from the enzyme, attempts were made to regain activity by addition of TPN and of various metal ions, including Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} ions, but they met with no success.

From the evidence of absorption spectroscopy, the end product of enzymic oxidation was the same for each substrate and gave a well-defined absorption maximum at 276 mμ. Hitherto, such a compound has not been implicated as a product of aromatic-ring fission and its chemical structure is at present under investigation by Professor B. Lythgoe. The isolation of each 100 mg. of pure substance for this purpose entails the use of extracts derived from 40 l. of culture, since the protocatechuic acid from which it is produced itself inhibits oxidation at concentrations higher than 9 μmoles/ml. The compound forms colourless prisms from water, m.p. 235° (decomp.); its solubility in water at 15° is about 20 mg./ml., it is very soluble in hot water and in cold ethanol and sparingly soluble in ether and chloroform. It contains no phenol or enol groups, analysis gives $(C_7H_6O_6)_n$ and alkaline titration indicates two carboxyl groups per molecule if $n = 1$. When it is formed from protocatechuic acid with the uptake of 1 mole of O_2/mole of substrate, little or no CO_2 is evolved.

Extracts precipitated with $(NH_4)_2SO_4$ as described, and then redissolved and dialysed against distilled water for 3 hr., lost their ability to catalyse the oxidation of protocatechuic acid. Activity was partially regained on addition of 2×10^{-4} M-$FeSO_4,7H_2O$ and completely regained at 10^{-3} M-$FeSO_4,7H_2O$; spectroscopic examination also showed accumulation of the compound absorbing maximally at 276 mμ. Activity was not restored by addition of Fe^{3+}, Mn^{2+}, Mg^{2+}, Co^{2+} or Zn^{2+} ions, all at 10^{-3} M.

Oxidation of derivatives of p-*cresol*

Whole cells which had been grown on *p*-cresol oxidized 4-methylcatechol [Fig. 5 (I)] and protocatechuic aldehyde (II) at the same initial rate as *p*-cresol and protocatechuic acid and with final uptakes of O_2 indicative of complete oxidation. However, although 3:4-xylenol (IV), 2:4-xylenol (VII) and 4-methylresorcinol (X) were rapidly oxidized, only 1·5 mole of O_2 was taken up per mole of substrate in each. This consumption of O_2 would be explained if reactions (*b*), (*c*) and (*d*) occurred (Fig. 5), with the accumulation of substitution products of *p*-hydroxybenzoic acid that the cells could not oxidize.

Cells harvested from 10 l. of *p*-cresol medium were resuspended in 6 l. of a solution containing 2·4 g. of 3:4-xylenol (IV) and 30 g. of KH_2PO_4, adjusted to pH 7 with NaOH, and were aerated at 30°. After 18 hr., when a small sample gave an orange precipitate with 2:4-dinitrophenylhydrazine, 3 l. of the bacterial suspension was Seitz-filtered, ether-extracted and the ethereal solution dried overnight with anhydrous Na_2SO_4. After evaporation to dryness at 40°, the white residue was re-

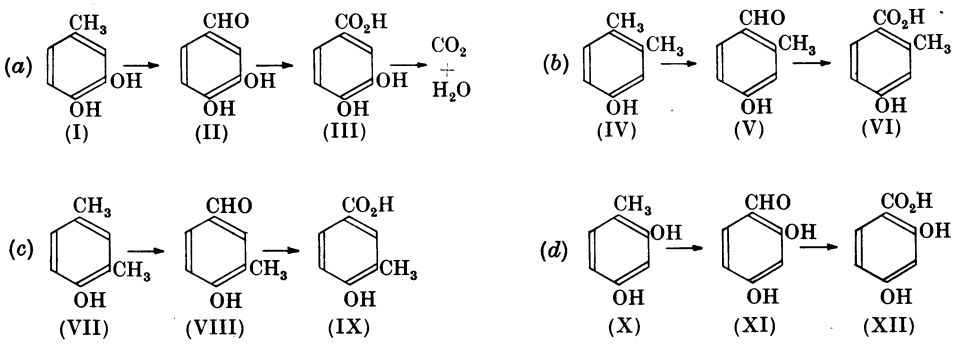

Fig. 5

Table 1. *Maximum values of absorption spectra of aldehydes and acids*

Compounds were dissolved in M/15 phosphate buffer (sodium and potassium salts, pH 6·98).

Compound ...	V	VI	VIII	IX	XI	XII
$\lambda_{max.}$ (mμ)	284	242	289, 337	250	280, 326	248, 292
$10^{-3}\,\epsilon_{max.}$	13·7	8·1	11·2, 4·5	12·6	12·0, 17·0	11·1, 5·2

crystallized three times from water and dried over $CaCl_2$ to give colourless plates, m.p. 108°; Heilbron & Bunbury (1943) give m.p. 110° for 4-hydroxy-2-methylbenzaldehyde (V) (Found: C, 70·5; H, 5·8. $C_8H_8O_2$ requires C, 70·6; H, 5·9). The remaining 3 l. of bacterial suspension after aeration for a further 27 hr. was Seitz-filtered and then extracted with ether. The ethereal solution was dried, the ether removed, the white residue recrystallized four times from water and the colourless crystals were dried over $CaCl_2$; m.p. 176–177°; Heilbron & Bunbury (1943) give m.p. 177–178° for 4-hydroxy-2-methylbenzoic acid (VI) (Found: C, 59·6; H, 5·4. $C_8H_8O_3,\frac{1}{2}H_2O$ requires C, 59·6; H, 5·6). By alkali titration, assuming the acid to be monobasic, the mol.wt. was 163; $C_8H_8O_3,\frac{1}{2}H_2O$ requires 161.

Cells grown with *p*-cresol and suspended in phosphate buffer containing 2:4-xylenol (VII) were treated similarly and from a sample removed at 18 hr. a compound was isolated which gave a 2:4-dinitrophenylhydrazone and formed colourless crystals from water, m.p. 118°. Heilbron & Bunbury (1943) give m.p. 118° for 4-hydroxy-3-methylbenzaldehyde (VIII) (Found: C, 70·6; H, 5·6. $C_8H_8O_2$ requires C, 70·6; H, 5·9). An acid crystallizing in colourless needles from water was obtained after further aeration of the culture. By alkali titration its mol.wt. was 161; m.p. 173–174°. Heilbron & Bunbury (1943) give 174–175° for 4-hydroxy-3-methylbenzoic acid (IX) (Found: C, 59·5; H, 5·6. $C_8H_8O_3,\frac{1}{2}H_2O$ requires C, 59·6; H, 5·6).

These isomeric aldehydes and acids are not readily obtainable and their absorption spectra are apparently not on record. To facilitate the study of enzymic reactions in which they participate, the absorption spectra of the compounds so isolated were plotted, together with those for resorcyl aldehyde (XI) and 2:4-dihydroxybenzoic acid (XII) which were available commercially. Molecular-extinction coefficients at wavelengths for maximum absorption are given in Table 1 for compounds dissolved in phosphate buffer, pH 6·98; extinction coefficients are influenced by changes in pH values. Fig. 6 shows the absorption spectra of the aldehydes and acids whose isolation has been described.

For whole cells, reactions (*d*) were shown to proceed, as for (*b*) and (*c*), with the formation of an aldehyde (XI) later converted into the acid (XII); but in this case the compounds were identified by their absorption spectra and not by isolation.

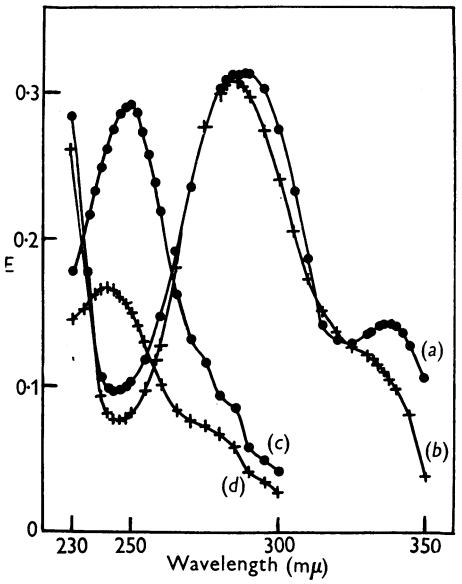

Fig. 6. Absorption spectra of aldehydes and acids. Each compound was dissolved in M/15 phosphate buffer, pH 6·98, to give a concentration of 0·02–0·03 mM. (*a*) 4-Hydroxy-3-methylbenzaldehyde; (*b*) 4-hydroxy-2-methylbenzaldehyde; (*c*) 4-hydroxy-3-methylbenzoic acid; (*d*) 4-hydroxy-2-methylbenzoic acid.

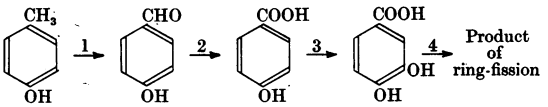

Fig. 7

Cell-free extracts oxidized aldehydes V, VIII and XI to their acids with the uptake of 0·5 mole of O_2/mole of substrate and with the shifts in absorption maxima anticipated. Benzaldehyde was not oxidized. After precipitation of extracts with $(NH_4)_2SO_4$, each aldehyde required the presence of DPN for oxidation; TPN was not an effective substitute.

DISCUSSION

Compounds in the reaction sequence illustrated (Fig. 7) were oxidized with the calculated uptake of oxygen in the presence of crude cell-free extracts.

Whole cells grown on *p*-cresol oxidized this substrate as rapidly as the other compounds shown, but with cell-free extracts *p*-cresol was oxidized at a slower rate. That a factor involved in reaction 1 was removed during the preparation of soluble extracts was suggested by the observation that oxidation of *p*-cresol was accelerated by addition of 'particles' from material in the well-defined pink-coloured layer that deposited over cell debris during centrifuging of crushed *Pseudomonas*. These particles carried a cytochrome system and appear to be very similar to the 'coarse-particle fraction' obtained by Stanier, Gunsalus & Gunsalus (1953) from *Pseudomonas fluorescens*, which was shown to catalyse the oxidation of mandelic acid and benzaldehyde. However, there is no direct evidence for the separate existence of these 'particles' inside the cell. Examination by the electron microscope showed a range of sizes which may arise from the disruption of a cellular structure, as Weibull (1953) has suggested for other insoluble-'particle' fractions. The possibility that *p*-hydroxybenzyl alcohol is an intermediate in reaction 1, as suggested by Smith *et al.* (1952), was not tested because of the difficulty of obtaining a pure specimen.

Gunter (1953) has shown that extracts of *Ps. fluorescens* adapted to oxidize *p*-hydroxymandelic acid catalyse reaction 2 but she did not investigate cofactor requirements. After precipitation with ammonium sulphate our preparations converted *p*-hydroxybenzaldehyde into *p*-hydroxybenzoic acid only on addition of DPN. The system is similar to that involved in the conversion of benzaldehyde into benzoic acid studied by Gunsalus, Stanier & Gunsalus (1953), who found two dehydrogenases in their extracts, one requiring DPN and the other TPN. However, although our extracts converted three other aldehydes into their acids in the presence of DPN, TPN could not be substituted and benzaldehyde was not oxidized. The preparations of either Gunsalus *et al.* (1953) or of Gunter (1953) did not oxidize the respective substrates (mandelic acid and *p*-hydroxymandelic acid) beyond benzoic or *p*-hydroxybenzoic acids; in each the next reaction involves hydroxylation, to give catechol or protocatechuic acid respectively. Our crude extracts, which catalyse reaction 3, appear to be the first cell-free system from bacteria to effect such a hydroxylation (see Stanier, 1955). Activity was lost on precipitation with ammonium sulphate and was not recovered by the addition of various cofactors, including DPN and TPN, which have been implicated in hydroxylation systems of mammalian origin (Udenfriend & Cooper, 1952; Mitoma, Posner, Reitz & Udenfriend, 1956). It is possible that the enzyme for reaction 3 is labile and may not survive this treatment, for Mitoma (1956) has shown that phenylalanine hydroxylase from rat livers consists of two fractions, one of which is labile.

The chemical structure of the compound arising from reaction 4 has not yet been elucidated. It is produced by union of 1 mole of protocatechuic acid with 1 mole of oxygen, without loss of carbon dioxide, in accordance with its empirical formula $C_7H_6O_6$. From extracts of *Ps. fluorescens* that converted protocatechuic acid into 3-oxoadipic acid, MacDonald, Stanier & Ingraham (1954) prepared protocatechuic oxidase, which catalyses the conversion of protocatechuic acid, with the uptake of 1 mole of oxygen/mole of substrate, into *cis-cis*-β-carboxymuconic acid without loss of carbon dioxide. More recently Gross, Gafford & Tatum (1956) have prepared from *Neurospora crassa* two enzymes, the first converting protocatechuic acid into *cis-cis*-β-carboxymuconic acid and the second converting this acid into its lactone; a delactonizing enzyme, or enzymes, then converts β-carboxymuconolactone into 3-oxoadipic acid with loss of carbon dioxide. By use of these enzymes, Gross *et al.* (1956) prepared β-carboxymuconolactone and showed that it was not attacked by extracts of *Ps. fluorescens* able to convert protocatechuic acid into 3-oxoadipic acid. Experiments with ^{14}C-labelled protocatechuic acid confirmed that in *Ps. fluorescens* this conversion occurs by reactions different from those in *Neurospora*. Apart from *cis-cis*-β-carboxymuconic acid, therefore, the compounds involved in the conversion of protocatechuic acid into 3-oxoadipic acid by *Ps. fluorescens* are not yet known; one of them may be the compound isolated as the end product of the reactions which our cell-free extracts catalyse. Further, the enzyme responsible for cleavage of the aromatic nucleus may differ from the protocatechuic oxidase of Stanier & Ingraham (1954) since we had little difficulty in showing that preparations which oxidized protocatechuic acid required Fe^{2+} ions as cofactor. Two other enzymes that catalyse similar fissions of aromatic nuclei also have this requirement, namely pyrocatechase (Suda, Hashimoto, Matsuoka & Kamahora, 1951) and homogentisic acid oxidase (Ravdin & Crandall, 1951); but no cofactor requirement could be found for the protocatechuic oxidase of Stanier & Ingraham (1954).

From reactions in which derivatives of *p*-cresol participated, conclusions can be drawn about substrate specificities of the enzymes involved. When derivatives contained a second methyl group (IV, VII) cells grown at the expense of *p*-cresol oxidized only the methyl group in the position *para* to the hydroxyl group. Various aldehydes (II, V, VIII, XI) with hydroxyl groups in position 4 were oxidized by whole cells or cell-free extracts to the corresponding acids; but benzaldehyde was not attacked. Of the compounds tested, aromatic-ring fission resulted

only when 3:4-dihydroxybenzoic acid was produced as an intermediate (I, II); 2:4-dihydroxybenzoic acid (XII) was not oxidized; and although crude extracts catalysed the introduction of a hydroxyl group into position 3 in *p*-hydroxybenzoic acid before ring fission, substitution products of this acid (VI, IX, XII) were not so attacked and nor was phenol. Evidently, attachment of an additional group to the aromatic nucleus of *p*-cresol or *p*-hydroxybenzaldehyde may not inhibit oxidation of the side chain; but the hydroxylation enzyme appears to act upon *p*-hydroxybenzoic acid specifically and not upon its derivatives.

SUMMARY

1. Cell-free extracts obtained from a species of *Pseudomonas* catalysed the oxidation of *p*-cresol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and protocatechuic acid. The amounts of oxygen utilized supported evidence from experiments with whole cells that the compounds participated in a reaction sequence in that order.

2. *p*-Hydroxybenzaldehyde was isolated as 2:4-dinitrophenylhydrazone after freeze-dried cells had oxidized *p*-cresol. A pink insoluble fraction from centrifuging of disintegrated cells carried a cytochrome system and increased the rate of oxidation of *p*-cresol by cell-free extracts.

3. Extracts that catalysed the conversion of *p*-hydroxybenzaldehyde into *p*-hydroxybenzoic acid required diphosphopyridine nucleotide (DPN) as cofactor. Other aromatic aldehydes with a hydroxyl group in position 4 also required DPN for conversion into the corresponding acids.

4. The enzyme catalysing hydroxylation of *p*-hydroxybenzoic acid was relatively labile, and, after treatment of extracts with ammonium sulphate, *p*-hydroxybenzoic acid accumulated when *p*-cresol and *p*-hydroxybenzaldehyde were oxidized. Specificity requirements of this enzyme were also apparently more stringent, since 2:4-xylenol, 3:4-xylenol and 4-methylresorcinol were oxidized to derivatives of *p*-hydroxybenzoic acid, but no further.

5. Ferrous ions were required for the oxidation of protocatechuic acid by extracts with an uptake of 1 mole of oxygen/mole of substrate. The end product of the reaction has been isolated, but it does not appear to be identical with any compound hitherto implicated in the biological fission of aromatic nuclei.

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