Oxidative Metabolism of Naphthalene by Soil Pseudomonads

THE RING-FISSION MECHANISM

BY J. I. DAVIES AND W. C. EVANS
Department of Biochemistry and Soil Science, University College of North Wales, Bangor

(*Received* 7 *October* 1963)

It has been known since the work of Tausson (1950) that the soil harbours micro-organisms that oxidatively metabolize polynuclear aromatic hydrocarbons derived from petroleum crude oil (Beerstecher, 1954). Several species of bacteria that grow in a naphthalene–mineral salts medium were subsequently described by Gray & Thornton (1928). The biochemistry of this process was first studied by Strawinski & Stone (1943), who isolated salicylic acid from naphthalene cultures of a soil pseudomonad; Walker & Wiltshire (1953), in addition, isolated D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene, and showed by the sequential-induction technique that both were intermediates in the metabolism of this hydrocarbon. Substitution of the naphthalene nucleus at C-8 by a halogen (chlorine or bromine) or a methyl group had little effect on the dissimilation of the unsubstituted ring; the corresponding salicylate and diol intermediates were isolated in every case (Walker & Wiltshire, 1955; Canonica, Fiecchi & Treccani, 1957). These results made it likely that C-4 of the naphthalenediol became the carboxyl C of salicylic acid; Walker & Evans (1952) had already shown that *Pseudomonas* spp. converted salicylate into catechol before ring-fission.

Murphy & Stone (1955) found that 1,2-naphthaquinone accumulated, eventually in toxic amounts, in naphthalene cultures of their strain of *Pseudomonas* when grown in media devoid of added ferrous and magnesium salts. Early attempts failed to show sequential induction to 1,2-dihydroxynaphthalene, the compound likely to be both the precursor of 1,2-naphthaquinone and the intermediate before ring-fission. However, Fernley & Evans (1958), by taking precautions to ensure its purity and guarding against non-enzymic quinone formation from this unstable catechol derivative, obtained positive results. Naphthalene-grown cells of their *Pseudomonas* sp. readily metabolized 1,2-dihydroxynaphthalene. From naphthalene cultures they also isolated coumarin, and detected small amounts of *o*-coumaric acid and melilotic acid. That 1,2-dihydroxynaphthalene was the actual derivative undergoing cleavage was finally established when a Fe^{2+} ion-dependent enzyme preparation from naphthalene-grown bacteria was shown to act on this substrate with the uptake of approximately three oxygen atoms and the liberation of one carbon dioxide molecule/molecule of substrate; from the reaction mixture coumarin was isolated. On the basis of these results Fernley & Evans (1958) advanced a tentative pathway of oxidative dissimilation of naphthalene as follows: Naphthalene → naphthalenediol → 1,2-dihydroxynaphthalene → (*o*-carboxy-*cis*-cinnamate) → *o*-coumarinate → salicylate → catechol → known pathways.

This scheme involved the postulate that *o*-carboxy-*cis*-cinnamate was the immediate ring-fission product, resulting from cleavage between the two hydroxyl groups. Yet, cells grown on naphthalene were not sequentially induced to metabolize two synthetic samples of this acid; oxygen uptakes observed with coumarin, *o*-coumarate and melilotate were also not sufficiently high to warrant the assumption of their role as intermediates, if strict adherence to this criterion was insisted on.

In view of these discrepancies, a detailed investigation of the enzymology of ring-fission of 1,2-dihydroxynaphthalene was undertaken. A preliminary report of the new cleavage mechanism described in the present paper has already appeared (Davies & Evans, 1962).

EXPERIMENTAL

Organisms. A fluorescent Gram-negative stumpy rod, classified tentatively as a member of the Pseudomonadaceae, was isolated from garden soil by elective culture on naphthalene–mineral salts media, by using a Collins & Sims (1956) soil perfuser. Subsequently, other strains known to decompose naphthalene were examined (N.C.I.B. cultures no. 8858 and no. 8859 obtained from the Torry Research Institute, Aberdeen).

The basal inorganic salts medium had the following composition (g./l.): $(NH_4)_2SO_4$, 1; K_2HPO_4, 1; $MgSO_4,7H_2O$, 0·3; $CaCl_2$, 0·1; $FeCl_3$, 0·02; adjusted to pH 7. Naphthalene, previously sterilized, was suspended in sterile mineral salts medium, subjected to ultrasonic homogenization and added at a concentration of 0·1% in the final medium. Liquid cultures (100 ml.) were grown in conical flasks fitted with baffles on a rotary shaker at 25°. Large-scale cultures (10–50 l.) were grown under forced aeration for 4 days, the small cultures being used as inocula. Cells were harvested at 13 000*g* in a bench-model compressed-air-driven

Sharples Super centrifuge fitted with a cellophan lining to the rotor; they were washed with 10 vol. of Na_2HPO_4–KH_2PO_4 buffer, pH 7 (0·05 M), and either used immediately or stored at −15° until required.

Cell-free extracts. Cell suspensions (1 g. wet wt. of cells) in 10 ml. of Na_2HPO_4–KH_2PO_4 buffer, pH 7 (0·05 M), were disintegrated in the M.S.E.–Mullard ultrasonic disintegrator at 0–4°. The cell-free extract was obtained by centrifuging at 20 000*g* for 20 min. at 0° and discarding the precipitate.

Materials. 1,2-Dihydroxynaphthalene, m.p. 102°, was prepared by the method of Stenhouse & Groves (1877) from 1,2-naphthaquinone (Eastman Kodak Ltd.), carefully purified by treatment with charcoal and recrystallized from CS_2 until the needles were colourless and the melting point sharp.

o-Carboxycinnamic acid, m.p. 175°, was prepared by the method of Leupold (1901), and the other isomer, m.p. 205°, as described by Greenspan (1947). Each of these acids, when heated above the melting point, is quantitatively lactonized to phthalidylacetic acid, m.p. 156°.

o-Carboxybenzoylacetic acid, m.p. 90°, was synthesized according to the method of Gabriel & Michael (1878) and Gabriel & Neumann (1893).

(±)-*trans*-1,2-Dihydro-1,2-dihydroxynaphthalene was a gift from Professor E. Boyland, Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London, S.W. 3.

Coumaraldehyde, m.p. 126°, was synthesized as described by Pauly & Wascher (1923).

o-Coumaric acid, m.p. 207–208° (decomp.), was obtained from coumarin (British Drug Houses Ltd.) by the method of Fittig & Ebert (1884). Melilotic acid, m.p. 82–83°, was obtained by the reduction of *o*-coumaric acid with sodium amalgam.

o-Carboxyphenylpropionic acid, m.p. 167°, was prepared according to the directions of Meyer, Philipps, Ruppert & Schmitt (1928).

Benzotetronic acid, m.p. 206°, was purchased from L. Light and Co.

The compound alleged to be 2-carboxybenzopyrilium perchlorate, m.p. 235° (effervescence), was prepared according to the method of Le Fèvre (1934).

o-Cresol, saligenin, catechol and naphthalene were products of British Drug Houses Ltd.

Thiamine pyrophosphate, β-NAD, NADP, coenzyme A, ATP and thioctic acid were all obtained commercially.

Microanalyses were by Weiler and Strauss, Oxford.

Estimations. Spectrophotometric determinations were carried out in the Unicam SP. 500 with silica cuvettes of 1 cm. light-path. Protein was estimated by the method of Warburg & Christian (1942).

Manometric experiments were conducted in the conventional Warburg constant-volume apparatus at 30°.

Acid production during enzymic reactions was measured by an apparatus consisting of an electrometric-titration assembly, automatic titrator and recording unit (Radiometer Ltd., Copenhagen). The reaction vessel was maintained at 30° by using a Circotherm (Braun, Melsungen, West Germany) circulating water from a bath.

The model 137 Infracord spectrophotometer (Perkin–Elmer Ltd.) was used to obtain infrared-absorption spectra of the regions 2·5–15 μ, a pressed KCl medium being used in every case.

Chromatography. The following solvents were used with Whatman no. 54 paper: (*a*) ethanol–aq. ammonia (sp.gr. 0·88)–water (16:1:3, by vol.); (*b*) sodium formate (5%, w/v)–formic acid (200:1, v/v); (*c*) propan-2-ol–water–ammonia (sp.gr. 0·88) (20:2:1, by vol.); (*d*) butan-1-ol–ethanol–ammonia (0·5 N) (7:1:2, by vol.); (*e*) butan-1-ol–ethanol–water (7:1:2, by vol.). Ascending or descending chromatography was employed to suit the purpose in hand.

Electrophoresis. Paper electrophoresis was carried out on Whatman no. 54 paper in pyridine (25 ml.)–acetic acid (10 ml.) buffer made up in 2·5 l. of water, by using a potential gradient of 13 v/cm.

Detection reagents. A mercury lamp (253·7 mμ) was used to locate substances that absorbed or fluoresced in ultraviolet light. Phenolic compounds gave characteristic colours after being sprayed with spotting reagents, e.g. Folin–Ciocalteu reagent, or diazotized *p*-nitroaniline followed by sodium carbonate (10%, w/v). Acids were detected by spraying with bromocresol green followed by dipping in aniline–xylose reagent (Smith, 1958). Chromatograms were passed through a solution of 2,4-dinitrophenylhydrazine (100 mg./100 ml. of 2 N-HCl) to develop visible spots from carbonyl compounds.

2,4-Dinitrophenylhydrazones fluoresce in ultraviolet light, and are visible. However, the sensitivity was increased by spraying the air-dried chromatograms with 10% (w/v) Na_2CO_3–N-NaOH (1:1, v/v). Fresh authentic samples of 2,4-dinitrophenylhydrazones of oxo acids were used as markers, since older samples streaked.

RESULTS

Sequential-induction experiments

By using young naphthalene-grown cells, the sequential-induction results of Fernley & Evans (1958) were confirmed in their entirety for the organism used in the present investigation. These included the strong induction to low concentrations of 1,2-dihydroxynaphthalene (VII; see Scheme 2) and the negative results for the two forms of *o*-carboxycinnamic acid (m.p. 175° and 205° respectively).

In addition to the above there was induction to D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene (VI), salicylaldehyde (XII), salicylate (XIII) and catechol (XIV); *o*-coumaraldehyde, coumarin, *o*-coumarate and melilotate regularly gave oxygen uptakes in excess of control cells, but the test was not as convincing with these latter substrates. No significant oxygen uptake was observed with *o*-carboxybenzoylacetate, *o*-carboxycinnamate, *o*-carboxyphenylpropionate, phthalidylacetate and phthalate. Fig. 1 illustrates some of the results.

In view of this very definite sequential-induction pattern, it was decided to focus attention on the actual method of ring-cleavage of 1,2-dihydroxynaphthalene, by using cell-free enzyme preparations.

Oxidation of 1,2-dihydroxynaphthalene

Properties of 1,2-dihydroxynaphthalene oxygenase. Fernley & Evans (1958) had already demonstrated the presence in naphthalene-grown cells of an oxygenase that cleaved the hydroxylated ring with simultaneous oxygen consumption. There were, however, experimental difficulties with this enzyme action. 1,2-Dihydroxynaphthalene is spontaneously oxidized very rapidly in aqueous solution to 1,2-naphthaquinone (VIII); approx. 20% oxidation/min. takes place at pH 6·5 in a solution containing 0·025 μmole of this substrate/ml. It is stable in the solid state or when dissolved in anhydrous solvents.

The enzyme solution is not easily assayed, since, to avoid the accumulation of 1,2-naphthaquinone, it is necessary to use sufficient enzyme to complete the cleavage of 1,2-dihydroxynaphthalene in 1–2 min. at pH 6·5. An approximate estimate of the activity of a cell-free extract was obtained as follows: serially-diluted cell-free extracts, in a total volume of 3 ml. of disodium hydrogen phosphate–potassium dihydrogen phosphate buffer, pH 6·5 (0·05 M), were incubated in test tubes at 30°. To each tube was added 1,2-dihydroxynaphthalene (1 μmole/ml.) which had been precipitated on to a small piece of filter paper from an anhydrous acetone solution. The activity of the enzyme solution was indicated by observing the greatest dilution that was still capable of preventing the formation of the yellow colour due to 1,2-naphthaquinone. By this method it was consistently found that the addition of ferrous sulphate (final concn. 1 mM) increased the activity of the enzyme 30–50-fold. Crude cell-free enzyme preparation (0·1 ml.; 12 mg. of protein/ml.) in the presence of ferrous sulphate (1 mM) usually metabolized 1 μmole of 1,2-dihydroxynaphthalene/ml. The enzyme is fairly stable, losing little activity on standing at 2–4° for 3 days. However, after dialysis against running tap water at 4° for 24 hr. there was a considerable decrease in activity, in spite of the addition of the requisite amount of ferrous sulphate.

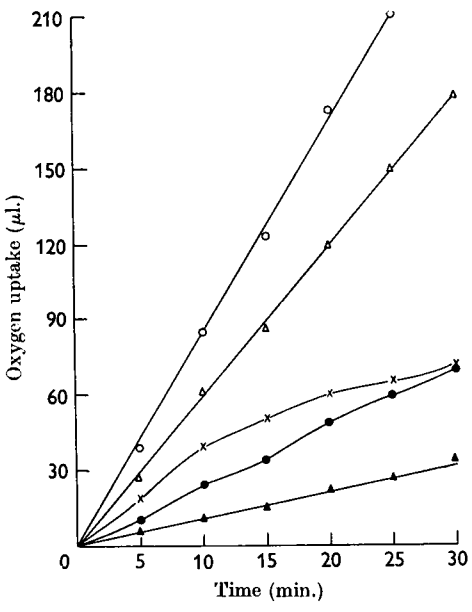


Fig. 1. Oxygen consumption by naphthalene-grown cells in the presence of salicylaldehyde (△), salicylate (○), coumarin (●), *cis-o*-hydroxybenzalpyruvate (×) or no added substrate (▲). Each Warburg flask contained cell suspension (0·5 ml.; approx. 15 mg. dry wt./ml.) and substrate (2 μmoles in 0·5 ml.; added from the side arm). The solutions above were made up in NaH_2PO_4–K_2HPO_4 buffer, pH 7 (0·05 M), which was also used to make up the total volume to 3·0 ml. The centre well contained 0·2 ml. of KOH (4 N).

Gaseous exchange during the action of the oxygenase on 1,2-dihydroxynaphthalene. 1,2-Dihydroxynaphthalene was oxidized by this oxygenase preparation with the consumption of 1 mole of oxygen/mole of substrate. No carbon dioxide was evolved; this is contrary to the results obtained by Fernley & Evans (1958) with an enzyme preparation from another organism.

Characterization of the initial ring-fission product. Four quantities of 1,2-dihydroxynaphthalene (30 mg. each) were added, with shaking, at 5 min. intervals to the standard enzyme solution (20 ml.; 12 mg. of protein/ml.) in 500 ml. of disodium hydrogen phosphate–potassium dihydrogen phosphate buffer, pH 6·5 (0·05 M), containing ferrous sulphate (1 mM), and incubated at 30°. Then, 5 min. after the final addition of substrate, the reaction mixture was extracted with ether (4 × 100 ml.) to remove neutral components. The solution was then acidified to pH 2·5 with 10% (w/v) sulphuric acid, extracted with ether (8 × 100 ml.) and the solvent dried with anhydrous sodium sulphate. After removal of the sodium sulphate by filtration, solid anhydrous barium chloride was added to the ether solution to remove traces of SO_4^{2-} ions (which, as shown below, hasten the decomposition of the ring-fission product). The filtered ether extract was concentrated to 1 ml., and 72% (w/v) perchloric acid (50 mg.) added. On being shaken, the perchloric acid phase became intensely green and deposited small needle-shaped yellowish green crystals. These were spun off, washed several times with anhydrous ether, and traces of solvents removed in the vacuum desiccator.

The perchlorate derivative had m.p. 228° (effervescence) (Found by microanalysis: C, 42·9; H, 2·8. $C_{10}H_7ClO_7$ requires C, 43·7; H, 2·6%).

The original ring-fission product was obtained spontaneously on dissolving the perchlorate crystals

in water or buffer, as shown by its absorption spectrum and its enzymic conversion into salicylaldehyde (see below).

2,4-Dinitrophenylhydrazone of the ring-fission product. The perchlorate of the ring-fission product (15 mg.) was treated with a slight excess of 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid and the mixture left to stand at 30° overnight. The red solid, which separated slowly, was collected and dissolved in ethyl acetate, washed with water and the solvent removed. It was triturated with benzene, then dissolved in ether and precipitated out of this solvent by light petroleum (b.p. 40–60°) to give a red powder, m.p. 211–219° (Found by microanalysis: C, 47·9; H, 3·7; N, 15·2. $C_{16}H_{12}N_4O_7$ requires C, 51·6; H, 3·3; N, 15·1%). The 2,4-dinitrophenylhydrazone derivative was shown by electrophoresis in pyridine–acetic acid–water buffer, pH 5·3 (5:2:500, by vol.), to consist of two fractions of slightly different mobility, but both were acidic since they moved towards the anode under these conditions. Under acid conditions the spots were orange and yellow respectively, whereas both became deep-red in 10% (w/v) sodium hydroxide. They were assumed to be isomeric forms.

Conversion of the ring-fission product into coumarin and o-coumaric acid. The crystalline yellowish green perchlorate (5 mg.) was dissolved in 2 ml. of 0·1N-sodium hydroxide, 0·1 ml. of 30% (w/v) hydrogen peroxide was added and the reaction mixture was kept overnight at room temperature, when the yellow colour disappeared. The solution was adjusted to pH 7 with 10% (w/v) sulphuric acid and steam-distilled; the distillate possessed the characteristic smell of coumarin, and had $\lambda_{max.}$ 277 mμ with a point of inflexion at 312 mμ. On being boiled with alkali, acidified and extracted with ether, it was converted into *o*-coumaric acid which had R_F 0·47 and R_F 0·20 in solvents (*b*) and (*c*), identical in every way with an authentic sample. The residue left after steam-distillation was made acid to Congo Red, extracted with ether and, after traces of water had been removed, the solvent was evaporated. The white crystalline residue was recrystallized from ethanol–water mixture, collected and dried; it had m.p. 206–208°, undepressed on admixture with an authentic specimen of *o*-coumaric acid.

The ring-fission product was also converted quantitatively into coumarin on standing at room temperature in 50% (w/v) sulphuric acid. This was isolated by steam-distillation after neutralization, through ether extraction of the distillate and recrystallization of the residue after the removal of solvent, from ethanol–water; it had m.p. 69°, undepressed on admixture with an authentic sample.

Properties of the ring-fission product. The perchlorate of the ring-fission product, as isolated from the enzyme action, has the following properties: (*a*) When the crystals are dissolved in water, a positive phenol reaction is given with the Folin–Ciocalteu reagent, and a red colour with diazotized *p*-nitroaniline. (*b*) The ultraviolet-absorption and visible-absorption spectra at various pH values are shown in Fig. 2. The compound is colourless in neutral and moderately acid aqueous solution (N-hydrochloric acid), with $\lambda_{max.}$ 213, 257 and 298 mμ; in strongly acid solution (6N-hydrochloric acid) it forms a yellow cation with $\lambda_{max.}$ 253 and 343 mμ. In alkali (N-sodium hydroxide) it gives an intense-yellow anion with $\lambda_{max.}$ 238, 306 and 420 mμ. (*c*) The infrared-absorption spectrum of the perchlorate (potassium chloride disk) is shown in Fig. 3. (*d*) When the perchlorate is dissolved in water, extracted with ether and the solvent dried and removed, there is left the free unstable oxo acid. It has an infrared-absorption spectrum shown in Fig. 4, with the absence of the absorption peaks due to the perchlorate ion (1040 and 1120 cm.$^{-1}$; cf. Fig. 3).

The properties of the ring-fission product of 1,2-dihydroxynaphthalene, namely its ready conversion into coumarin on oxidation with peroxide, the presence of carboxylic, carbonyl and phenolic groupings, together with the analysis of the perchlorate and its absorption characteristics, provide strong evidence that the compound is *o*-hydroxy-

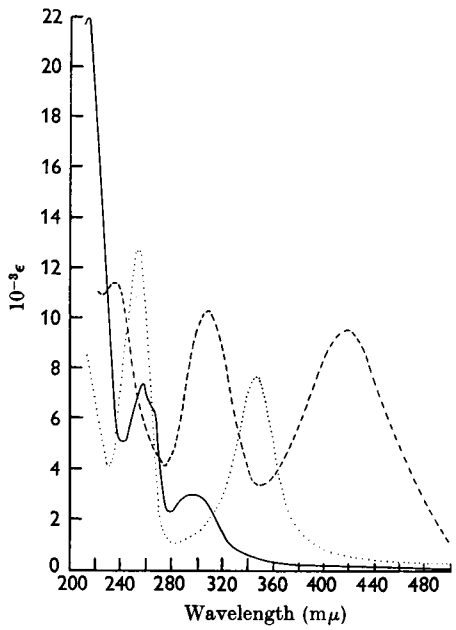


Fig. 2. Ultraviolet-absorption spectra of the various forms of *cis-o*-hydroxybenzalpyruvate: cation (X) (· · · · · · · ·, in 6N-HCl); hemiacetal (I) (——, in buffer, pH 7); anion (II) (- - - -, in 0·1N-NaOH).

benzalpyruvate. That the carbonyl grouping is involved in cyclization under acidic conditions is suggested by the presence of only one peak in both infrared-absorption spectra (1720–1730 cm.$^{-1}$; Figs. 3 and 4) due to the carboxyl group, and by its ultraviolet-absorption spectrum which shows that a very pronounced loss of conjugation occurs at neutral pH as compared with that in alkali (Fig. 2). Moreover, the ease of coumarin formation from this *o*-hydroxybenzalpyruvate indicates that it has the *cis*-configuration. The well-characterized perchlorate is envisaged as the pyrilium salt (X, p. 259); the cyclic non-cationic form is represented as the hemiacetal (I, p. 257). Cleavage of the oxygen bridge in alkali releases a carbonyl group to yield the anionic form represented as (II, p. 257), which explains the extinction maximum at 420 mμ.

Condensation of salicylaldehyde and pyruvic acid. Benzalpyruvate is formed in good yield by the condensation of benzaldehyde and pyruvate in methanolic potassium hydroxide (Reimer, 1931; Friedmann, 1931). When salicylaldehyde and pyruvic acid were mixed in equimolecular proportions with methanolic potassium hydroxide and allowed to stand for 14 days, no condensation took place; this was also noted by Le Fèvre (1934). Under acid conditions, however, reaction occurred, giving two products, as follows.

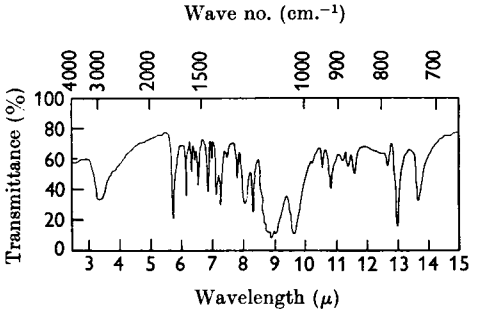


Fig. 3. Infrared-absorption spectrum of *cis*-*o*-hydroxybenzalpyruvic acid perchlorate.

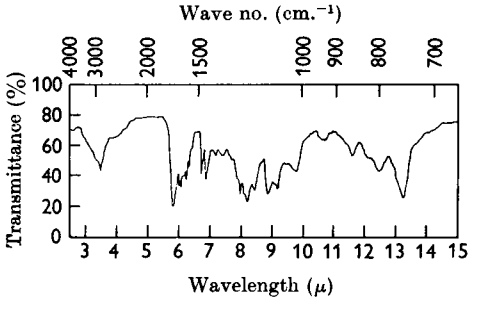


Fig. 4. Infrared-absorption spectrum of the hemiacetal form of *cis*-*o*-hydroxybenzalpyruvic acid.

Salicylaldehyde (6 g.) and pyruvic acid (4·5 g.) were mixed, cooled to 0° and saturated with dry hydrogen chloride. After 24 hr. at room temperature, the crystals that had been deposited were separated from the syrupy dark supernatant liquid by filtration, after dilution with ether. After being washed with ether (4 × 10 ml.), ethanol (2 × 5 ml.) and finally ether again (2 × 10 ml.), the greenish-yellow crystals (1·7 g.) had m.p. 202–203° (decomp.). This compound is referred to below.

A second compound was isolated from the supernatant as follows. The ethereal filtrate from the condensation was shaken with water (50 ml.) to which 2N-sodium hydroxide was cautiously added until the pH had risen to 7. Unchanged salicylaldehyde was removed from the aqueous phase by repeated extraction with ether (4 × 50 ml.). The aqueous phase was reacidified with 2N-hydrochloric acid and extracted with ether (3 × 50 ml.); this ether extract was washed once with water (10 ml.) before being shaken with saturated lead acetate (5 g.), when a yellow precipitate appeared. After adjustment to pH 6 with 2N-sodium hydroxide the lead salt was filtered and washed several times with water to free it from any unchanged pyruvic acid. The lead salt was then suspended in water (50 ml.), decomposed by 2N-sulphuric acid and the free acid extracted into ether (3 × 50 ml.). Evaporation of the ether yielded an oil which failed to crystallize, but which yielded an unstable 2,4-dinitrophenylhydrazone, a quinoxalinol derivative, m.p. 161–165° (decomp.), after recrystallization from chloroform, and a semicarbazone derivative, m.p. 159–163°, after recrystallization from methanol–xylene mixture.

The properties of this oily reaction product were identical with those of a compound formed when *cis*-*o*-hydroxybenzalpyruvate (the ring-fission product) was boiled for a few minutes in aqueous solution at neutral pH; this changed the R_F values in solvents (*a*) and (*b*) from 0·98 and 0·95 (i.e. that of *cis*-*o*-hydroxybenzalpyruvate) to 0·71 and 0·67 respectively. The chromatographic spots of R_F 0·71 and 0·65 gave an orange fluorescence in ultraviolet light, a positive phenol reaction with the Folin–Ciocalteu reagent, and a 2,4-dinitrophenylhydrazone. On elution they had $\lambda_{max.}$ 298 and 343 mμ in aqueous solution at pH 7, changing to $\lambda_{max.}$ 308 and 420 mμ in alkali (Fig. 5).

The 2,4-dinitrophenylhydrazones formed both from the product obtained by boiling *cis*-*o*-hydroxybenzalpyruvate in buffer and from the oily condensation product of salicylaldehyde and pyruvate gave well-defined spots of equal mobilities on electrophoresis in pyridine–acetic acid buffer, pH 5·3.

Oxidation of the oily condensation product with alkaline hydrogen peroxide gave *o*-coumaric acid,

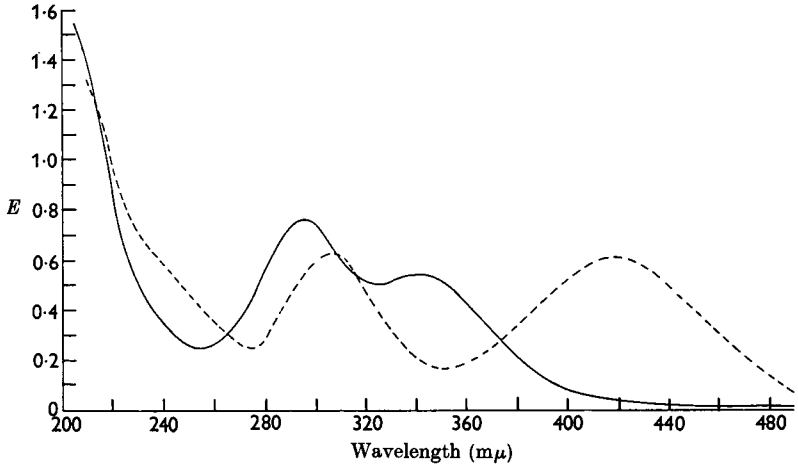


Fig. 5. Ultraviolet-absorption spectrum (extinctions) of *trans-o*-hydroxybenzalpyruvate in NaH_2PO_4–K_2HPO_4 buffer, pH 7 (——), and 0·1 N-NaOH (- - - -).

m.p. 208°, in a similar manner to the ring-fission product, *cis-o*-hydroxybenzalpyruvate. Perchloric acid (72 %) added to a cooled concentrated ether solution of the condensation product gave an intense-red aqueous phase, but no crystalline derivative separated.

The ease of formation of this compound from *cis-o*-hydroxybenzalpyruvate, the conditions of condensation between salicylaldehyde and pyruvate, its oxidation to coumarate by alkaline peroxide, together with the other properties described, indicate that it is most probably the *trans*-isomer of the ring-fission product, *cis-o*-hydroxybenzalpyruvate.

Le Fèvre (1934) synthesized a yellow-green crystalline compound which he claimed to be 2-carboxybenzopyrylium perchlorate, m.p. 230–235° (decomp. and effervescence), by condensing salicylaldehyde and pyruvic acid in the presence of perchloric acid, together with hydrochloric acid-saturated ether at 0°. This was repeated, and yielded a product, m.p. 230–235°, sparingly soluble in water. A mixed melting point with the enzymically prepared *cis-o*-hydroxybenzalpyruvic perchlorate showed a depression, indicating that the two compounds were not identical. They also differed in their solubility properties.

When the compound described by Le Fèvre (1934) was dissolved in dilute alkali and reacidified with 2 N-hydrochloric acid, crystals were deposited, which were identical with those of m.p. 202–203° (see above) (Found: C, 59·5; H, 3·16 %; no Cl^- or ClO_4^- ions present). The ultraviolet-absorption and infrared-absorption spectra are shown in Figs. 6 and 7 respectively, the latter apparently showing the presence of two carbonyl groups ($\lambda_{max.}$ 1700 and 1760 cm.$^{-1}$). The absence of any further evidence as

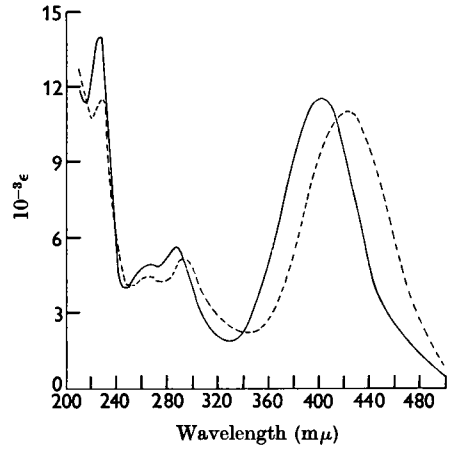


Fig. 6. Ultraviolet-absorption of the compound, m.p. 202–203° (decomp.) (see the text) in NaH_2PO_4–K_2HPO_4 buffer, pH 7 (0·05 M) (——), and 2 N-HCl (- - - -). The positions of the maxima in neutral solution were unchanged in 2 N-NaOH.

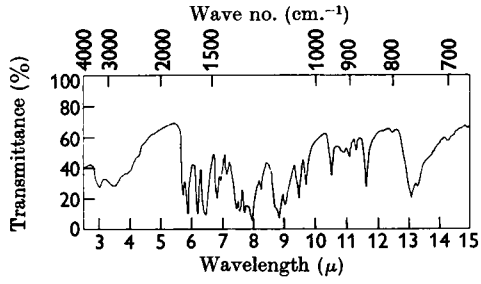


Fig. 7. Infrared-absorption spectrum of compounds m.p. 202–203° (decomp.) (see the text).

to the structure of Le Fèvre's (1934) 2-carboxybenzopyrylium perchlorate precludes any useful comparison between it and the enzymically isolated *cis-o*-hydroxybenzalpyruvic perchlorate, for which structure considerable other supporting evidence exists.

Production of salicylaldehyde by the action of a cell-free enzyme system on 1,2-*dihydroxynaphthalene and* cis-o-*hydroxybenzalpyruvate.* When 1,2-dihydroxynaphthalene was incubated with a dilute cell-free extract as described above, partial conversion into *o*-hydroxybenzalpyruvate took place, but another product could be extracted from the neutral reaction mixture with ether. This non-acidic compound was phenol-positive (Folin–Ciocalteu reagent) and gave a brown colour with diazotized *p*-nitroaniline. It was steam-volatile, and the ultraviolet-absorption spectra had $\lambda_{max.}$ 256 and 325 mμ (in phosphate buffer, pH 7) changing to $\lambda_{max.}$ 227, 266 and 377 mμ in alkali, and identical with salicylaldehyde. It gave a 2,4-dinitrophenylhydrazone derivative as orange crystals from ethanol, m.p. 251–253° (decomp.), undepressed by admixture with authentic salicylaldehyde 2,4-dinitrophenylhydrazone.

Enzymic conversion of o-*hydroxybenzalpyruvate into salicylaldehyde.* When *trans-o*-hydroxybenzalpyruvate (IV; see Scheme 1) (derived either by isomerizing the *cis-o*-hydroxybenzalpyruvate or by chemical synthesis) is dissolved in 0·1 M-sodium hydrogen carbonate, the *trans*-yellow anion (III) appears instantaneously, whereas under similar conditions it takes several hours for the conversion of the hemiacetal (I) into the corresponding *cis*-yellow anionic configuration (II).

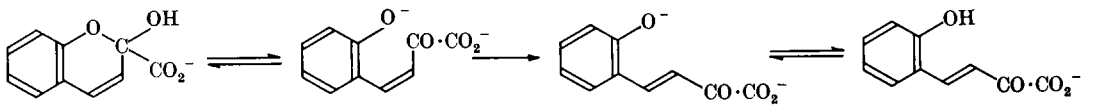
The following experiment compares the rates of enzymic production of salicylaldehyde from the two isomers under standard conditions. Solutions of the *cis-* and *trans*-isomers in disodium hydrogen phosphate–potassium dihydrogen phosphate buffer, pH 7 (0·05 M) were adjusted so that the extinction at 300 mμ was 0·44 in both cases. Cell-free enzyme (0·04 ml.; 20 mg. of protein/ml.) was added to each, and the decrease in extinction at 300 mμ with time was followed. The *trans-o*-hydroxybenzalpyruvate peak at 298 mμ completely disappeared in 10 min., to be replaced by that of salicylaldehyde at 325 mμ, whereas that due to the *cis*-form persisted almost unchanged. The experiment was repeated by using the substrates and enzyme in 0·1 M-sodium hydrogen carbonate: again the *trans*-isomer was converted into salicylaldehyde (sodium salt: $\lambda_{max.}$ 377 mμ), whereas the *cis*-form remained unchanged. However, if the *cis-o*-hydroxybenzalpyruvate was allowed to stand for several hours in this solution, the *cis*-yellow anion appeared, which was rapidly converted into salicylaldehyde by the same enzyme solution.

The inability of intact naphthalene-grown cells to metabolize the hemiacetal form (I) may account for the results obtained in sequential-induction experiments (Fig. 1). A rapid initial oxygen uptake was consistently followed by a rate similar to that of cells in the absence of added substrate. Complete metabolism of small quantities of the *cis*-anion in equilibrium with the hemiacetal may account for the initial rapid attack.

Properties of the enzyme converting o-*hydroxybenzalpyruvate into salicylaldehyde.* By using the above spectrophotometric-assay procedure, it was shown that the enzyme is very stable in solution; standing in the cold room (2–4°) or dialysis against running tap water for 4 days caused very little loss of activity. It is insensitive to 1 mM-potassium cyanide.

Cell-free enzyme solution (0·2 ml.; 12 mg. of protein/ml.) diluted to 3 ml. with disodium hydrogen phosphate–potassium dihydrogen phosphate buffer, pH 6·5 (0·05 M), containing ferrous sulphate (1 mM), was allowed to act on 1,2-dihydroxynaphthalene (1 μmole) in the presence of the following cofactors (each at 0·5 mM), both individually and in combination: NAD, NADP, CoA, thioctic acid, thiamine pyrophosphate and ATP. The accumulated *cis-o*-hydroxybenzalpyruvate was determined by removing salicylaldehyde (by ether extraction), making the residual aqueous phase alkaline with potassium hydroxide and measuring its extinction at 420 mμ. The results showed that none of these additions affect the quantity of *cis-o*-hydroxybenzalpyruvate accumulated. Thus the enzymic reaction converting *cis-o*-hydroxybenzalpyruvate into salicylaldehyde is independent of any diffusible cofactors.

Fate of the side chain of o-*hydroxybenzalpyruvate.* Manometric and titrimetric experiments at pH 8·0 showed that the conversion of *o*-hydroxybenzalpyruvate into salicylaldehyde entails no oxygen



(I) (II) (III) (IV)

Scheme 1. Possible transformations of *cis-o*-hydroxybenzalpyruvate.

uptake and no change in the total acidity of the solution. The nature of the C_3 side-chain fragment is therefore at issue.

The neutral reaction mixture in which *cis-o*-hydroxybenzalpyruvate was accumulated by enzyme action on 1,2-dihydroxynaphthalene (see above) was extracted with ether to remove salicylaldehyde, one of the known products of the enzyme action. The aqueous residue was freeze-dried and then triturated with ether saturated with 2N-hydrochloric acid. The acid–ether extract was allowed to react with excess of aqueous 2,4-dinitrophenylhydrazine reagent and the acidic hydrazones were subjected to electrophoresis in pyridine–acetic acid buffer, pH 5·3, and paper chromatography. Spots were obtained with R_F 0·50 and 0·54 in solvent (*c*), 0·42 in solvent (*d*) and 0·36 in solvent (*e*), as found for an authentic sample of pyruvic 2,4-dinitrophenylhydrazone. The simultaneous presence of *cis-o*-hydroxybenzalpyruvic 2,4-dinitrophenylhydrazone caused some streaking of chromatograms and rendered determinations of pyruvate difficult, but it appeared that the concentration of the latter may not have equalled that of salicylaldehyde which is also formed in the reaction, possibly because of its further metabolism.

Conversion of salicylaldehyde into salicylate by a dehydrogenase. The oxidation of salicylaldehyde to salicylate by the cell-free enzyme system specifically required the addition of NAD (but not NADP). A cytochrome system was present, so the reaction was assayed manometrically, and shown to involve the consumption of one atom of oxygen/molecule of substrate. The oxidation product, salicylate, was detected by chromatography in solvents (*b*) and (*c*), R_F 0·70 and 0·59 respectively, its purple fluorescence and ultraviolet-absorption spectrum of the eluted spot. Neither saligenin nor *o*-cresol is oxidized by this system; they therefore cannot be intermediates in salicylaldehyde formation from 1,2-dihydroxynaphthalene.

Further metabolism of salicylate. Whole-cell suspensions of naphthalene-grown cells after storage at −15° for several weeks, on incubation with naphthalene, salicylate or catechol at neutral pH, developed a yellow colour in the supernatant solution. The accumulated compound is extracted into ether only at acid pH (when it is colourless); it gives an orange–red colour with *o*-dianisidine (aldehyde test) and an acidic 2,4-dinitrophenylhydrazone derivative. Its ultraviolet-absorption spectra in acid ($\lambda_{max.}$ 317 mμ) and alkali ($\lambda_{max.}$ 373 mμ) are identical with those of α-hydroxymuconic semialdehyde (Dagley, Evans & Ribbons, 1960). This compound was also produced on incubation of catechol with the standard cell-free extract of naphthalene-grown cells.

DISCUSSION

Scheme 2 shows the proposed pathway of naphthalene dissimilation by the soil pseudomonads used in the present study.

The demonstration that *cis-o*-hydroxybenzalpyruvate is the immediate ring-fission product of 1,2-dihydroxynaphthalene establishes both a new intermediate and the position of ring-cleavage in the oxidative metabolism of naphthalene by a soil pseudomonad. Moreover, all the other naphthalene-utilizing bacteria since examined employ the same mechanism, i.e. oxidative fission of the bond adjacent to the hydroxyl groups of 1,2-dihydroxynaphthalene, between the angular C and C-1 of the naphthalene nucleus. This method is analogous to that described for the metabolism of tryptophan through the so-called 'quinoline' pathway, employed by some *Pseudomonas fluorescens* strains; here kynurenic acid is formed, which then gives the diol, 7,8-dihydro-7,8-dihydroxykynurenic acid (Taniuchi & Hayaishi, 1963); this is then dehydrogenated to 7,8-dihydroxykynurenate, followed by breakage of the bond between C-8 and the adjacent angular C, to give 5-(γ-carboxy-γ-oxopropenyl)-4,6-dihydroxypicolinic acid (Kojima, Itada & Hayaishi, 1961).

cis-o-Hydroxybenzalpyruvate may undergo oxidative decarboxylation to coumarin (XI) even during removal of the solvent, on the water bath, from a sodium sulphate-dried acid–ether extract of the oxo acid. The coumarin isolated from naphthalene cultures, and incubation mixtures of 1,2-dihydroxynaphthalene with cell-free extracts, reported by Fernley & Evans (1958), we now believe to be an artifact produced in this way, since it has often been repeated unless precautions were taken to prevent it.

The ease with which coumarin is produced from the new ring-fission product indicates a *cis*-configuration of the double bond; however, isolation of a small amount of coumaric acid from the products of oxidation with peroxide in alkaline solution may indicate that some inversion took place during this step.

cis-o-Hydroxybenzalpyruvic acid, isolated enzymically as the crystalline perchlorate, has some interesting properties. In aqueous solution it can exist in three forms depending on the pH.

The structure shown for the greenish yellow cationic form (X) (2-carboxybenzopyrilium perchlorate, m.p. 228°) is consistent with its C and H microanalysis, the infrared-absorption spectrum (Fig. 3) and the ease with which it is converted into the colourless form (I) on decreasing the acidity, e.g. merely dissolving the crystals in water. The lack of complete conjugation suggested by the ultraviolet-absorption spectrum of the colourless

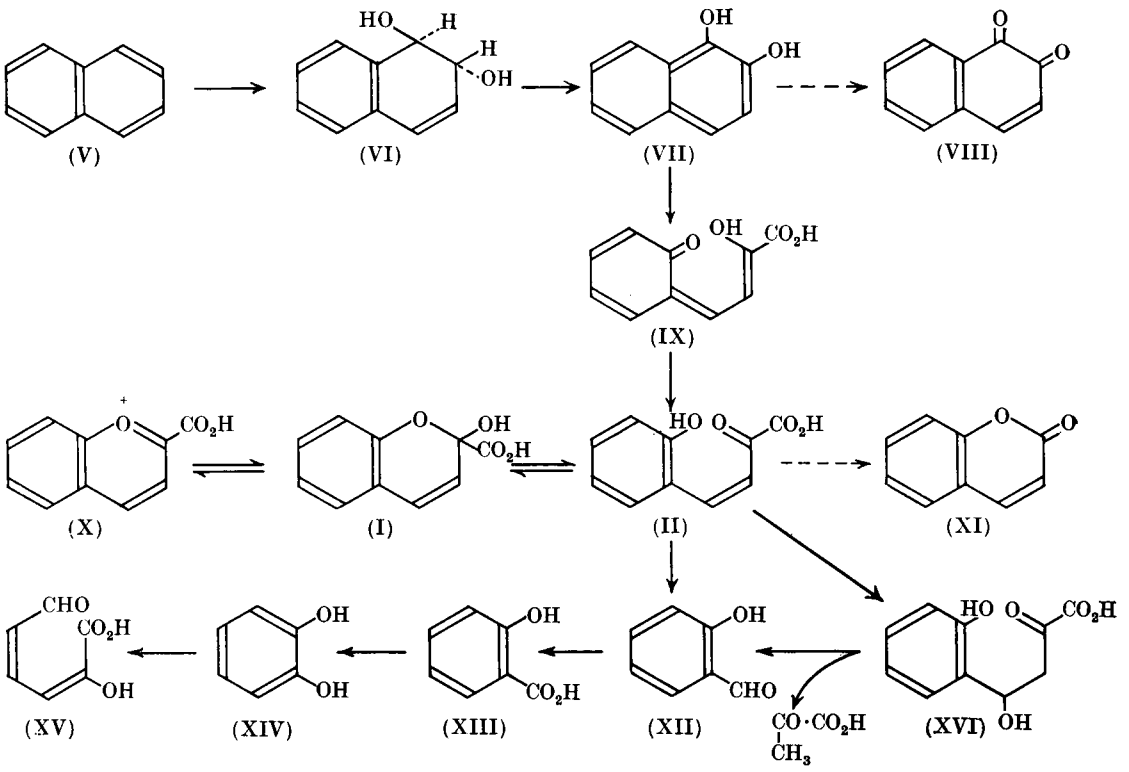
form is explained by the proposed hemiacetal structure. The infrared-absorption spectrum is consistent with the latter (Fig. 4), together with the observed slow rate of formation of its 2,4-dinitrophenylhydrazone in 2N-hydrochloric acid and the time required for conversion into the yellow anionic form (II) in alkali, a phenomenon not observed with the *trans*-isomer, which is incapable of cyclization. The yellow anion (II) shows extensive electron delocalization, and has an ultraviolet-absorption spectrum similar to that of the sodium salt of *o*-hydroxycinnamaldehyde (coumaraldehyde) ($\lambda_{max.}$ 233, 306 and 416 mμ) and the anionic form of *trans*-*o*-hydroxybenzalpyruvate.

The colourless hemiacetal form of *cis*-*o*-hydroxybenzalpyruvic acid (I) is not directly attacked by cell-free extracts, and apparently only with difficulty by whole cells; it therefore does not represent a true intermediate. The yellow anionic form (II) is, however, rapidly attacked by crude cell-free extracts, giving salicylaldehyde and a fragment tentatively identified as pyruvate (2,4-dinitrophenylhydrazone derivative). The actual intermediate is therefore likely to be this anionic form (II) or its quinonoid structure (IX). The side-chain split involves no diffusible cofactors or gaseous exchange, and may be a retro-aldol cleavage.

This would occur through hydration of the double bond to give γ-hydroxy-γ-*o*-hydroxyphenyl-α-oxobutyrate (XVI), followed by cleavage (cf. ketohydroxybutyrase; Hift & Mahler, 1952).

The salicylaldehyde dehydrogenase is NAD-specific; Gunsalus, Stanier & Gunsalus (1953*b*) separated two benzaldehyde dehydrogenases from *Pseudomonas fluorescens* grown on (±)-mandelate with specific cofactor requirements for NAD and NADP respectively. The conversion of salicylate into catechol by whole cells has long been established (Walker & Evans, 1952); Katagiri, Yamamoto & Hayaishi (1962) separated salicylate hydroxylase from the catechol-cleavage enzyme and showed it to require $NADH_2$ or $NADPH_2$ and $FADH_2$ as electron donors. All the organisms tested which metabolize naphthalene, when grown on this substrate, employ the method of catechol dissimilation through α-hydroxymuconic semialdehyde (Dagley & Stopher, 1959).

As reported by Fernley & Evans (1958), acid–ether extracts of naphthalene cultures yield coumarin, and chromatographic evidence for the



Scheme 2. Proposed pathway of naphthalene metabolism by soil pseudomonads.

presence of *o*-coumaric acid and melilotic acid was obtained. Sequential-induction data excluded all the *o*-carboxyphenyl derivatives from being intermediates: *o*-carboxybenzoylacetate, *o*-carboxycinnamate, *o*-carboxyphenylpropionate, phthalidylacetate and phthalate. But several of the *o*-hydroxyphenyl series could be metabolized without an apparent lag phase by naphthalene-grown cells, e.g. coumarin, coumaric acid, melilotic acid, etc.

It has now been shown that non-enzymic conversion of *cis*-*o*-hydroxybenzalpyruvate into coumarin takes place during chemical isolation procedures, but the origin of some of the other *o*-hydroxyphenyl derivatives cannot be dismissed in this way.

The fate of an aliphatic side-chain substituent on an aromatic ring during microbial metabolism shows some diversity according to the organism used and the cultural conditions. Thus, with mandelate, degradation of the side chain via benzoylformate to benzoate occurs before ring-fission (Gunsalus, Stanier & Gunsalus, 1953*a*). If the substituent is reduced to acetate, preferential attack on the aromatic nucleus occurs by hydroxylation and ring-cleavage, with the side chain remaining intact, e.g. phenylacetate is converted via homogentisate into maleoylacetoacetate in some organisms (Chapman & Dagley, 1962), and into 3,4-dihydroxyphenylacetate immediately before ring-fission in others (P. J. Chapman & S. Dagley, personal communication). In the phenylpropane series, cinnamate and phenylpropionate both give rise to 2,3-dihydroxyphenylpropionate, via melilotate, before cleavage of the ring (Coulson & Evans, 1959; Lloyd, 1961; P. J. Chapman & S. Dagley, personal communication). However, in still cultures, cinnamate gives rise to acetophenone (isolated as the 2,4-dinitrophenylhydrazone derivative), which is metabolized probably through benzoate, in the organism which under conditions of forced aeration produces 2,3-dihydroxyphenylpropionate, i.e. a completely different pathway (Fernley, 1959).

With the C_4 side chain of *cis*-*o*-hydroxybenzalpyruvate several possibilities exist for its disruption, in addition to the hydrolytic fission between C-3 and C-4, for which we have demonstrated the presence of the appropriate inducible enzymes, and which we believe to be the main pathway. There exists the possibility that such a potentially reactive side chain may be amenable to attack by constitutive enzymes; oxidative decarboxylation would give *o*-coumarinate, which in the presence of an isomerase (responsible for conversion of the *cis*-form into the *trans*-form, and well known in this field) could yield *o*-coumarate, to be metabolized via melilotate, and 2,3-dihydroxyphenylpropionate, followed by ring-fission. Ring-fission reactions possess attributes of specificity, by virtue of the limited possibilities that exist for the biochemical conversion of an aromatic ring into aliphatic metabolites. With aliphatic structures the possibility of diversity in their metabolic pathways is much increased.

SUMMARY

1. Naphthalene is oxidatively metabolized by soil pseudomonads through D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene to 1,2-dihydroxynaphthalene, which then undergoes ring-cleavage.

2. The immediate product of ring-fission is *cis*-*o*-hydroxybenzalpyruvate, isolated as the crystalline perchlorate. The Fe^{2+} ion-dependent oxygenase therefore disrupts the bond between the angular C and C-1 of the naphthalene nucleus.

3. Some properties of *cis*-*o*-hydroxybenzalpyruvate are described. Under alkaline conditions it exists as the open-chain *cis*-yellow anion, the real intermediate in the metabolic pathway. At neutral or slightly acid pH, cyclization to the hemiacetal form occurs; in strong acid it exists as the yellow pyrilium cation.

4. *trans*-*o*-Hydroxybenzalpyruvate is obtained by isomerization of the enzymically prepared *cis*-form, and by chemical synthesis. Both are converted without gaseous exchange into salicylaldehyde, and a fragment tentatively identified as pyruvate, by an enzyme system of an aldolase type isolated from the bacteria.

5. A NAD-specific dehydrogenase is also present in the cell-free extract that oxidizes the salicylaldehyde to salicylate. The latter is then oxidatively decarboxylated to catechol, which is completely dissimilated through α-hydroxymuconic semialdehyde.

6. All the naphthalene-utilizing micro-organisms so far examined adopt the pathway delineated above.

J. I. D. acknowledges a Research Studentship from the Agricultural Research Council. We also thank Dr D. R. Marshall of the Chemistry Department, University College of North Wales, Bangor, for helpful discussion on certain aspects of this work.

REFERENCES

Beerstecher, E. (1954). *Petroleum Microbiology*. New York: Elsevier Press Inc.

Canonica, L., Fiecchi, A. & Treccani, V. (1957). *R.C. Ist. lombardo*, **91**, 119.

Chapman, P. J. & Dagley, S. (1962). *J. gen. Microbiol.* **28**, 251.

Collins, F. M. & Sims, C. M. (1956). *Nature, Lond.*, **178**, 1073.

Coulson, C. B. & Evans, W. C. (1959). *Chem. & Ind.* p. 543.

Dagley, S., Evans, W. C. & Ribbons, D. W. (1960). *Nature, Lond.*, **188**, 560.

Dagley, S. & Stopher, D. A. (1959). *Biochem. J.* **73**, 16P.

Davies, J. I. & Evans, W. C. (1962). *Biochem. J.* **85**, 21P.
Fernley, H. N. (1959). Ph.D. Thesis: University of Wales (Bangor).
Fernley, H. N. & Evans, W. C. (1958). *Nature, Lond.*, **182**, 373.
Fittig, R. & Ebert, G. (1884). *Liebigs Ann.* **226**, 351.
Friedmann, E. (1931). *Helv. chim. acta*, **14**, 783.
Gabriel, S. & Michael, A. (1878). *Ber. dtsch. chem. Ges.* **10**, 1553.
Gabriel, S. & Neumann, A. (1893). *Ber. dtsch. chem. Ges.* **26**, 953.
Gray, P. H. & Thornton, H. G. (1928). *Zbl. Bakt.* **73**, 74.
Greenspan, F. P. (1947). *Industr. Engng Chem. (Anal.)*, **39**, 847.
Gunsalus, I. C., Stanier, R. Y. & Gunsalus, C. F. (1953*a*). *J. Bact.* **66**, 538.
Gunsalus, I. C., Stanier, R. Y. & Gunsalus, C. F. (1953*b*). *J. Bact.* **66**, 548.
Hift, H. & Mahler, H. R. (1952). *J. biol. Chem.* **198**, 901.
Katagiri, M., Yamamoto, S. & Hayaishi, O. (1962). *J. biol. Chem.* **237**, PC2413.
Kojima, Y., Itada, N. & Hayaishi, O. (1961). *J. biol. Chem.* **236**, 2223.
Le Fèvre, R. J. W. (1934). *J. chem. Soc.* p. 450.
Leupold, E. (1901). *Ber. dtsch. chem. Ges.* **34**, 2833.
Lloyd, G. T. (1961). Ph.D. Thesis: University of Wales (Bangor).
Meyer, F., Philipps, H., Ruppert, F. W. & Schmitt, A. Th. (1928). *Ber. dtsch. chem. Ges.* **61**, 1971.
Murphy. J. F. & Stone, R. W. (1955). *Canad. J. Microbiol.* **1**, 579.
Pauly, H. & Wascher, K. (1923). *Ber. dtsch. chem. Ges.* **56**, 603.
Reimer, M. (1931). *J. Amer. chem. Soc.* **53**, 3147.
Smith, I. (1958). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 279. London: W. Heinemann (Medical Books) Ltd.
Stenhouse, J. & Groves, C. E. (1877). *J. chem. Soc.* **32**, 53.
Strawinski, R. J. & Stone, R. W. (1943). *J. Bact.* **45**, 16.
Taniuchi, H. & Hayaishi, O. (1963). *J. biol. Chem.* **238**, 283.
Tausson, W. O. (1950). In *Basic Principles of Plant Bioenergetics (Collected Works)*, p. 48. Ed. by Maximov, N. A. Moscow: Academy of Sciences of the U.S.S.R.
Walker, N. & Evans, W. C. (1952). *Biochem. J.* **52**, xxiii.
Walker, N. & Wiltshire, C. H. (1953). *J. gen. Microbiol.* **8**, 273.
Walker, N. & Wiltshire, C. H. (1955). *J. gen. Microbiol.* **12**, 478.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.

Biochem. J. (1964) **91**, 261

Changes in the Activities of Urea-Cycle Enzymes after the Administration of Carbon Tetrachloride

BY PATRICIA McLEAN AND F. ROSSI
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1, and Institute of General Pathology (Unit 'G. Vernoni' of C.N.R.), University of Padua, Italy

(*Received* 2 *September* 1963)

The liver damage resulting from carbon tetrachloride poisoning leads to a number of metabolic changes among which is a profound disturbance of nitrogen metabolism. This disturbance is associated with a decline in the rate of incorporation of amino acids into protein (Smuckler, Iseri & Benditt, 1962), a decrease in amino acid activation (Rossi & Zatti, 1963*a*), an increase in the concentration of free ammonia in the liver (Gordon, 1959), and an increase in the concentrations of amino acids and ammonia in blood (Knauff & Windsheimer, 1960; Flores, Rosado, Torres & Soberón, 1962), as well as with a number of other changes in the nitrogenous composition of blood and urine.

It has also been observed that the activity of ornithine transcarbamoylase in the blood rises sharply after the administration of carbon tetrachloride (Reichard, 1959); at somewhat longer time-intervals, decreases in the activities of glutamate dehydrogenase (Rees & Sinha, 1960; Rees, Sinha & Spector, 1961) and glutamate–pyruvate transaminase have also been observed (Bengmark & Olsson, 1962).

In the expectation that such dramatic changes in amino acid metabolism would be reflected by parallel changes in the urea-forming systems of the liver, it seemed important to examine the effects of carbon tetrachloride poisoning on the entire sequence of enzymes involved in ureogenesis. Such an approach appeared to have the advantage that measurements of a series of enzymes linked in a metabolic sequence is of greater biological significance than measurements confined to individual enzymes. Flores *et al.* (1962) have studied the effects of carbon tetrachloride poisoning at 24 hr. and after 1 month on the activity of urea-cycle enzymes, and in the latter case have found striking decreases in activity.

The present experiments are concerned primarily with the short-term effects of carbon tetrachloride