Oxidative Metabolism of Phenanthrene and Anthracene by Soil Pseudomonads

THE RING-FISSION MECHANISM

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1. Phenanthrene is oxidatively metabolized by soil pseudomonads through *trans*-3,4-dihydro-3,4-dihydroxyphenanthrene to 3,4-dihydroxyphenanthrene, which then undergoes cleavage. 2. Some properties of the ring-fission product, *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid, are described. The Fe^{2+} -dependent oxygenase therefore disrupts the bond between C-4 and the angular C of the phenanthrene nucleus. 3. An enzyme of the aldolase type converts the fission product into 1-hydroxy-2-naphthaldehyde (2-formyl-1-hydroxynaphthalene). An NAD-specific dehydrogenase is also present in the cell-free extract, which oxidizes the aldehyde to 1-hydroxy-2-naphthoic acid. This is then oxidatively decarboxylated to 1,2-dihydroxynaphthalene, thus allowing continuation of metabolism via the naphthalene pathway. 4. Anthracene is similarly metabolized, through 1,2-dihydro-1,2-dihydroxyanthracene to 1,2-dihydroxyanthracene, in which ring-fission occurs to give *cis*-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid. The position of cleavage is again at the bond between the angular C and C-1 of the anthracene nucleus. 5. Enzymes that convert the fission product through 2-hydroxy-3-naphthaldehyde into 2-hydroxy-3-naphthoic acid were demonstrated. The further metabolism of this acid is discussed. 6. The Fe^{2+} -dependent oxygenase responsible for cleavage of all the *o*-dihydroxyphenol derivatives appears to be catechol 2,3-oxygenase, and is a constitutive enzyme in the *Pseudomonas* strains used.

Tausson (1928, see also 1950) isolated from oil-soaked soil of the Baku oilfields three strains of bacteria that attacked phenanthrene, namely *Bacillus phenanthrenicus bakiensis*, *Bacillus phenanthrenicus guricus* and *Bacterium phenanthrenicum*. These micro-organisms also metabolized a variety of other polynuclear aromatic hydrocarbons, including anthracene. Gray & Thornton (1928) and Sisler & Zobell (1947) demonstrated later that bacteria with these potentialities were of ubiquitous occurrence in soils and marine sediments respectively.

Rogoff & Wender (1957a) and Colla, Biaggi & Treccani (1957) initiated investigations towards understanding the biochemistry of these oxidative dissimilations. Their soil bacteria did not attack phenanthrene at the 9,10-bond (i.e. the so-called 'K region'; Pullman & Pullman, 1955) as postulated by Tausson, but was directed at an end ring. Thus Colla, Fiechi & Treccani (1959) isolated 3,4-dihydro-3,4-dihydroxyphenanthrene from cultures of a *Flavobacterium* metabolizing phenanthrene, and Rogoff & Wender (1957a) had already obtained

1-hydroxy-2-naphthoic acid from such cultures of a soil *Pseudomonas*. Both groups of workers showed that salicylate and catechol were later intermediates in this oxidative pathway, by the sequential-induction technique.

Results with an anthracene-utilizing strain also indicated end-ring attack, since 2-hydroxy-3-naphthoic acid was isolated from these cultures (Rogoff & Wender, 1957b). In addition, Colla *et al.* (1959) obtained a dihydroarene diol intermediate from anthracene cultures, and proposed a 1,2-configuration for it; these cells were also induced to metabolize salicylate and catechol.

Davies & Evans (1964) were able to ascertain the position of ring-cleavage in the microbial oxidative metabolism of naphthalene, the simplest polynuclear aromatic hydrocarbon. This takes place at the 1,2-dihydroxynaphthalene stage, by rupture of the bond between the angular C and C-1 to give *cis*-*o*-hydroxybenzalpyruvate (*cis*-4-*o*-hydroxyphenyl-2-oxobut-3-enoate).

In view of these findings, it appeared probable that the end-ring attack on phenanthrene and

anthracene occurs in an analogous manner to that of naphthalene. This would imply that 3,4-dihydroxyphenanthrene and 1,2-dihydroxyanthracene are intermediates, and that fission takes place at the bond between the angular C and C-4 and C-1 respectively. The results described below substantiate this hypothesis, and have already appeared in a preliminary communication (Fernley, Griffiths & Evans, 1964).

EXPERIMENTAL

Organisms. A strain of *Pseudomonas aeruginosa* (P_R), kindly provided by Dr M. H. Rogoff of the U.S. Bureau of Mines, Pittsburgh Coal Research Centre, Pa., U.S.A., capable of growing in a mineral salts medium together with either phenanthrene or anthracene (0.25%) as sole substrate, was used initially in this work. *Pseudomonas* strains P_F and P_G were isolated from garden soil after selective culture on naphthalene-mineral salts medium; it was found that these organisms could be adapted to grow on phenanthrene and anthracene (0.2%)-mineral salts medium in shake cultures.

The basal inorganic salts medium had the following composition (g./l.): $(NH_4)_2SO_4$, 1; K_2HPO_4 , 1; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.02; adjusted to pH 7. The hydrocarbon, previously sterilized, was suspended in a small volume of sterile mineral salts medium and subjected to ultrasonic homogenization before addition to the medium to a concentration of 0.2%. Liquid cultures (200 ml.) were grown in conical flasks fitted with baffles, on a rotary shaker at 25°, and used as inocula for large-scale cultures (10–50 l.) grown under forced aeration. The yield of cells from phenanthrene-adapted cultures was always much heavier than those from anthracene-adapted cultures. Cells were harvested at 13000g in a bench-model compressed-air-driven Sharples Super centrifuge fitted with a cellophane 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 -25° until required.

When it was discovered that naphthalene-grown cells of these strains contained the enzymes that acted on 3,4-dihydroxyphenanthrene and 1,2-dihydroxyanthracene, it was found more practicable to use them as source of enzymes in subsequent experiments.

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 MSE-Mullard ultrasonic disintegrator at 0–4°, or in the Hughes (1951) press. The cell-free extract was obtained by centrifuging at 20000g for 30 min. at 0° and discarding the precipitate. Further purification of the relevant enzyme system is described in the text.

Materials. Naphthalene, phenanthrene, anthracene, 2,3-dihydroxynaphthalene, 1-hydroxy-2-naphthoic acid, 2-hydroxy-3-naphthoic acid and catechol were commercial samples from British Drug Houses Ltd. (Poole, Dorset) and Koch-Light Labs. Ltd. (Colnbrook, Bucks.). β -NAD and NADP were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

3,4-Dihydroxyphenanthrene (morphol), m.p. 142°, was synthesized by the method of Werner (1902), Smith (1916) and Barger (1918) via the following route: phenanthrene $\rightarrow$ phenanthrene-3-sulphonic acid $\rightarrow$ 3-phenanthrol-4-aldehyde $\rightarrow$ morphol, and crystallized from water in white flakes; it is moderately stable even in aqueous media.

1,2-Dihydroxyanthracene, m.p. 160–162°, was obtained by reducing alizarin stepwise through deoxyalizarin to 1,2-dihydroxyanthracene, and recrystallized from carbon disulphide (Graebe & Thode, 1906; Hall & Perkin, 1923). Like 1,2-dihydroxynaphthalene (see Davies & Evans, 1964), it is rather unstable in aqueous media, and is better kept as the diacetyl derivative, from which it may be easily regenerated by hydrolysis with acetic acid and hydrochloric acid.

6,7-Benzocoumarin, m.p. 163° (Boehm & Profft, 1931), was synthesized from 2-hydroxy-3-naphthoic acid via the following intermediates: 2-acetoxy-3-naphthoyl chloride $\rightarrow$ 2-acetoxy-3-naphthaldehyde, followed by a Perkin condensation on this, which afforded the 6,7-benzocoumarin, and recrystallized from benzene.

7,8-Benzocoumarin, m.p. 141° (Bartsch, 1903), was prepared by condensing 1-naphthol with malic acid in the presence of sulphuric acid. It was recrystallized from acetone and finally ethanol.

1-Hydroxy-2-naphthaldehyde, m.p. 54–55°, was prepared by the method of Hamada (1952) from 2-naphthol by the Reimer-Tiemann reaction.

2-Hydroxy-3-naphthaldehyde, m.p. 99° (Boehm & Profft, 1931), was obtained as an intermediate in the synthesis of 6,7-benzocoumarin.

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, Denmark). The reaction vessel was maintained at 30° by using a Circotherm (Braun, Melsungen, West Germany) circulating water from a bath.

Chromatography. The following solvents were used with Whatman no. 54 paper: (a) sodium formate (5%, w/v)-formic acid (200:1, v/v); (b) butan-1-ol-water-aq. ammonia (sp.gr. 0.88) (20:2:1, by vol.); (c) ethanol-aq. ammonia (sp.gr. 0.88)-water (16:1:3, by vol.); (d) benzene-acetic acid-water (125:72:3, by vol.). Ascending or descending chromatography was employed, depending on their suitability.

Detection reagents. The Chromatolite lamp 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 10% (w/v) Na_2CO_3 . Aldehydes were detected by spraying with *o*-dianisidine in acetic acid (saturated). Acids were detected by spraying with bromocresol green followed by dipping in aniline-xylose reagent (Smith, 1958). α -Oxo acids showed up as yellowish-green-fluorescent spots under ultraviolet light after spraying with *o*-phenylenediamine in trichloroacetic acid (Dawson, Elliott, Elliott & Jones, 1959). Chromatograms were passed through a solution of 2,4-dinitrophenylhydrazine hydrochloride (100 mg./100 ml. of 2N-HCl) to develop visible spots from carbonyl compounds. 2,4-Dinitrophenylhydrazones absorb in ultraviolet light and are visible; however, the sensitivity was increased by spray-

Table 1. *Oxygen consumption by phenanthrene-grown cells with different substrates*

Each Warburg flask contained cell suspension (0.5 ml.; approx. 15 mg. dry wt./ml.) and substrate [2μ moles of (A) in 0.5 ml. added from side arm, or 1.5 μ moles of (B) that had been precipitated on to a small piece of filter paper from anhydrous acetone solution]. The solutions above were made up in $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 6.5 (0.05 M), which was also used to make up the total volume 3.0 ml. The centre well contained 0.2 ml. of 4 N-KOH.

Substrate	O ₂ taken up in first 10 min. (μ l.)
No added substrate	4
(A) 7,8-Benzocoumarin	7
(A) 1-Hydroxy-2-naphthoic acid	20
(A) 1-Hydroxy-2-naphthaldehyde	28
(B) 3,4-Dihydroxyphenanthrene	28
(B) 1,2-Dihydroxynaphthalene	45

ing the air-dried chromatograms with 10% (w/v) $\text{Na}_2\text{CO}_3\text{--N-NaOH}$ (1:1, v/v).

RESULTS

Phenanthrene metabolism

Sequential-induction experiments. By using phenanthrene-grown cells harvested towards the end of the logarithmic phase of growth, the sequential-induction results of Rogoff & Wender (1957a) were confirmed for all the organisms used in the present investigation. These were: immediate oxygen uptake with 1-hydroxy-2-naphthoic acid, salicylate and catechol; in addition there was a positive result with low concentrations of 3,4-dihydroxyphenanthrene, 1-hydroxy-2-naphthaldehyde and 1,2-dihydroxynaphthalene. 7,8-Benzocoumarin gave an oxygen uptake slightly above the control, but it was not convincingly positive. These results are shown in Table 1. 3,4-Dihydro-3,4-dihydroxyphenanthrene was not available to us at the time, but Colla *et al.* (1959) have shown by isolation and sequential-induction evidence that it is an intermediate in the pathway.

Detection of 1-hydroxy-2-naphthoic acid and 7,8-benzocoumarin from phenanthrene-adapted cultures. A phenanthrene-adapted culture (101.) of strain P_R after 48 hr. growth showed an accumulation of Folin-positive compounds; the cells were harvested, and the supernatant fluid was concentrated in a climbing-film evaporator at neutral pH, acidified to Congo red and continuously extracted with ether for 24 hr. The ether extract gave a solid on evaporation, which on chromatography in solvent (d) showed a spot, R_F 0.9, giving a dark-blue fluorescence under ultraviolet light, a purple-brown with diazotized *p*-nitroaniline-10% sodium carbonate and a deep

blue fading to purple-brown with diazotized *p*-nitroaniline-N-sodium hydroxide. Recrystallization from ethanol gave needles, m.p. 191° undepressed by an authentic sample of 1-hydroxy-2-naphthoic acid. This is in agreement with previous workers.

It was also noticed that phenanthrene-adapted cultures (101.) of strain P_R gave an instantaneous green colour with diazotized *p*-nitroaniline-10% sodium carbonate and a transient crimson with diazotized sulphanilic acid-10% sodium carbonate in the early phase of growth (24 hr. culture); the Folin reaction was only slightly positive at this stage. It was concentrated at neutral pH to 200 ml.; these colour reactions were now given more intensely, but on acidification they were abolished. The acidified concentrate was saturated with ether and extracted with benzene (2×50 ml.); the benzene extract, after being shaken with dilute sodium hydrogen carbonate solution to remove acidic components, was evaporated to dryness under reduced pressure. The small solid residue was dissolved in a little chloroform, diluted with an equal volume of light petroleum (b.p. $40\text{--}60^\circ$) and the precipitate discarded. The solvent phase was evaporated to a small bulk; chromatography in solvent (c) showed a spot, R_F 0.83, giving a green colour with diazotized *p*-nitroaniline-10% sodium carbonate and a blue fading to green with diazotized *p*-nitroaniline-N-sodium hydroxide. This spot had the same R_F and colour reactions as authentic 7,8-benzocoumarin. Elution of the chromatogram with water gave a solution with ultraviolet absorption spectrum identical with that of 7,8-benzocoumarin (see below).

From this evidence it appeared probable that the actual intermediate undergoing fission was 3,4-dihydroxyphenanthrene; attention was therefore directed towards the preparation of the cell-free ring-cleavage enzyme, as a first step towards a detailed study of this reaction.

Isolation and partial purification of the enzyme system that oxidatively cleaves 3,4-dihydroxyphenanthrene. (a) Assay procedure. The crude cell-free extract (0.5 ml.), prepared as described above (adjusted to contain 10 mg. of protein/ml. and referred to as fraction A), attacked 3,4-dihydroxyphenanthrene (1.5 μ mole) in disodium hydrogen phosphate-potassium dihydrogen phosphate buffer, pH 6.5 (0.05 M), to a total volume of 3 ml. in a Warburg apparatus, with simultaneous oxygen consumption and disappearance of the substrate. Since this catechol derivative is itself unstable in aqueous solution at pH 7 or above, forming the quinone, the substrate was precipitated on to a small piece of filter paper from an anhydrous acetone solution, and a pH of 6.5 maintained in the reaction mixture. The rate of oxygen uptake, with various amounts of crude enzyme solution of known protein concentration and a constant amount of substrate, under

the above conditions affords a rough measure of enzyme activity. This extract, however, carries the oxidation further than the initial cleavage step; separation of the ring-fission enzyme from other interfering components was accomplished as follows: Ammonium sulphate (5.77 g.) was added to fraction A (40 ml.) at 0° and the precipitate removed in the refrigerated centrifuge. The supernatant solution was treated with a further 3.73 g. of ammonium sulphate and the resulting precipitate (25–40% saturation) collected by centrifugation and redissolved in 40 ml. of phosphate buffer, pH 7 (0.05 M), previously saturated with nitrogen gas. This fraction B (3.2 mg. of protein/ml.) was free from the enzyme system active on the first product of ring cleavage of 3,4-dihydroxyphenanthrene. Dialysis against $\alpha\alpha'$ -bipyridyl (1 mM) in nitrogen-saturated phosphate buffer, pH 7.5 (0.01 M), overnight in the cold room at 2° resulted in an enzyme solution whose activity on 3,4-dihydroxyphenanthrene could be increased 100% by the addition of Fe^{2+} (1 mM). No other cation was found that could replace Fe^{2+} in this role. The sensitivity of the ring-fission enzyme to oxygen was evident if extraction and purification was carried out without taking precautions to displace the dissolved air with nitrogen; the preparations obtained were much less active. They could, however, be reactivated again by reduction with sodium borohydride (Taniuchi, Kojima, Kanetsuna, Ochiai & Hayaishi, 1962) as follows: 3 ml. of 'inactive' enzyme solution was placed in a Thunberg-Keilin tube together with sodium borohydride (3 mg.) in the side arm. It was evacuated with a high-vacuum pump until frothing ceased, and then tipped. After standing for 15 min. at room temperature under vacuum, the tube was opened and 0.1 ml. of 0.2 M-potassium dihydrogen phosphate added to readjust the pH to 7, and assayed. In many cases activity was restored to the level obtained when the utmost precautions had been taken in the preparation of the enzyme. Partial restoration of activity was also obtained on standing *in vacuo* in the presence of Fe^{2+} alone.

(b) *Oxidation of 3,4-dihydroxyphenanthrene by the ammonium sulphate-precipitated enzyme (fraction B).* A partially purified enzyme preparation (0.5 ml.; 3.2 mg. of protein/ml.) in the presence of Fe^{2+} (1 mM) oxidized 1.5 μmoles of 3,4-dihydroxyphenanthrene in a 3 ml. reaction mixture containing phosphate buffer, pH 6.5 (0.05 M), with the consumption of 1 mol. of oxygen/mol. of substrate. No carbon dioxide was evolved. A parallel enzyme action carried out in the automatic titrator showed that 1 carboxyl group was produced/mol. of substrate oxidized.

The product was colourless in acid solution (0.1 N-hydrochloric acid) with λ_{max} at 216, 262 and 300 $m\mu$; in alkali (0.1 N-sodium hydroxide) it gave

Fig. 1. Ultraviolet-absorption spectra in 0.1 N-NaOH (—) and in 0.1 N-HCl (---) of the ring-fission product of 3,4-dihydroxyphenanthrene.

an orange-brown anion with λ_{max} at 220, 312 and 465 $m\mu$ (Fig. 1). It was extracted into ether from acid solution, and from the ether into 4% sodium hydrogen carbonate. Reacidification and ether extraction followed by drying with anhydrous sodium sulphate and removal of traces of sulphate with anhydrous barium chloride gave an ether solution, suitable for chromatography after concentration to a small volume under reduced pressure. In solvent (a) one spot, R_F 0.33, was obtained, with the following properties: (i) under u.v. light (Chromatolite lamp) it shows up as a dull-yellow spot; (ii) exposure of the chromatogram to ammonia fumes produces a visible orange-brown colour; (iii) it reacts with 2,4-dinitrophenylhydrazine, indicating the presence of a keto group; (iv) it gives a rose-pink colour with diazotized *p*-nitroaniline, turning to blue-grey with sodium carbonate (10%). (v) a blue colour is given with the Folin-Ciocalteu phenol reagent; (vi) the *o*-phenylenediamine test for an α -oxo acid is positive.

2,4-Dinitrophenylhydrazone of the ring-fission product. Two 10 mg. quantities of solid 3,4-dihydroxyphenanthrene were added with shaking at 5 min. intervals to 50 ml. of the purified enzyme solution (3.2 mg. of protein/ml.) in disodium hydrogen phosphate-potassium dihydrogen phosphate buffer, pH 6.5 (0.05 M), containing ferrous sulphate (1 mM), and incubated at 30°. Then, 10 min. after the final addition of substrate, the reaction mixture was acidified to pH 2.5 with dilute hydrochloric acid (10%, w/v) and extracted with ether (5 × 100 ml.). The combined ether extract was shaken with sodium hydrogen carbonate (20 ml.; 4%, w/v) consecutively four times, and the combined bicarbonate solution acidified to Congo red with dilute hydrochloric acid. It was then treated with a slight excess of 2,4-dinitrophenylhydrazine in 2 N-hydrochloric acid and the mixture kept overnight in the cold room. The deep-red solid was collected, washed with 2 N-hydrochloric acid and then water, and finally dried in a vacuum desiccator, before recrystallization

from either benzene or ethanol, m.p. 170–175° (Found: C, 56.2; H, 3.1; N, 12.9. $C_{20}H_{14}N_4O_7$ requires C, 56.8; H, 3.3; N, 13.3%).

Electrophoresis in pyridine–acetic acid–water buffer, pH 5.3 (5:2:500, by vol.), showed the benzene-recrystallized specimen to consist of one major and one minor component of slightly different mobility; both were acidic since they moved towards the anode under these conditions, and were possibly isomeric forms.

Conversion of the 'ring-fission product' into 7,8-benzocoumarin. A concentrated acid–ether extract of a large-scale enzymic oxidation of 3,4-dihydroxyphenanthrene (20 mg., and 50 ml. of enzyme fraction B) was shaken with 10 ml. of 0.1 N-sodium hydroxide. The alkaline aqueous phase, containing the ring-fission product as the sodium salt, was separated from the neutral-ether fraction and treated with 0.2 ml. of 30% (w/v) hydrogen peroxide at room temperature. After about 30 min. the characteristic orange colour of the ring-fission product had disappeared; the solution was acidified with 3 N-sulphuric acid and allowed to stand another 30 min. before extraction with ether (3 × 100 ml.). The combined ether extract was shaken with 10 ml. of 4% sodium hydrogen carbonate solution to remove acidic components, and the neutral-ether fraction dried with anhydrous sodium sulphate. The ether extract showed ultraviolet-absorption $\lambda_{\max}$ at 265 and 275 m μ and $\lambda_{\min}$ at 245 m μ . Chromatography in solvents (b) and (c) showed one spot, R_F 0.94 and 0.83 respectively. It appeared bright blue under the Chromatolite lamp, and gave a blue colour with diazotized *p*-nitroaniline followed by N-sodium hydroxide, after some time. Evaporation of the neutral-ether extract gave a yellow solid that recrystallized from ethanol–water mixture in pale-yellow needles, m.p. 140–141° undepressed by an authentic sample of 7,8-benzocoumarin.

Structure of the ring-fission product of 3,4-dihydroxyphenanthrene: *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid. The mode of formation of the ring-fission product from 3,4-dihydroxyphenanthrene involving the consumption of 1 mol. of oxygen/mol., the appearance of 1 carboxyl group/mol. of substrate and the presence of a phenolic group indicate that cleavage has occurred between the angular C and C-4. Although it was possible to obtain virtually pure ethereal solutions of this compound, removal of the solvent invariably led to an oily residue that failed to crystallize; evolution of carbon dioxide and changes in its absorption spectrum indicated that the substance is unstable. The formation of a 2,4-dinitrophenylhydrazone derivative that gave the expected analysis, and the ease with which it gave 7,8-benzocoumarin on oxidation with hydrogen peroxide under alkaline conditions, strongly support the view that this new

ring-fission product is *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid. Its ultraviolet-absorption characteristics in acid and alkali are wholly consistent with this structure. The infrared spectrum is discussed below.

Condensation of 1-hydroxy-2-naphthaldehyde and pyruvic acid. When the conditions used by Reimer (1931) and Friedmann (1931) for the preparation of benzalpyruvic acid (3-benzylidenepyruvic acid) were applied, no condensation took place. This was analogous to the experience of Davies & Evans (1964) in their attempted synthesis of *cis*-o-hydroxybenzalpyruvate. Under acid conditions however, some reaction occurred. 1-Hydroxy-2-naphthaldehyde (18 mg.) and pyruvic acid (10 mg.) were dissolved in dry ether (sodium-dried) (20 ml.), cooled to 0° and saturated with dry hydrogen chloride gas. The original yellow colour became green. After standing for 48 hr. at room temperature, the ethereal solution was washed with water and carefully extracted with 4% sodium hydrogen carbonate to remove acidic components; the bicarbonate solution was made acid to Congo red and re-extracted with ether. Concentration of this ether extract to a small volume and chromatography in solvent (a) gave a spot, R_F 0.33, that was Folin-positive and reacted with 2,4-dinitrophenylhydrazine reagent. On elution, it had ultraviolet-absorption $\lambda_{\max}$ at 216, 262 and 300 m μ in acid, changing to $\lambda_{\max}$ 220, 312 and 465 m μ in alkali. These properties are identical with those of *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid obtained enzymically. Attempts to isolate the condensation product in crystalline form were unsuccessful.

Oxidation of 3,4-dihydroxyphenanthrene by the crude cell-free extract (fraction A). When 3,4-dihydroxyphenanthrene (20 mg.) was incubated with 50 ml. of the crude cell-free extract (fraction A) (10 mg. of protein/ml.) together with ferrous sulphate (1 mM) in phosphate buffer, pH 6.5 (0.05 M), and aerated, a different product to that obtained when using the purified enzyme (fraction B) was produced. The acid–ether extract of the incubation mixture was yellow, and only a trace of this colour was removed by 4% sodium hydrogen carbonate solution. It could, however, be extracted from the ether into 0.1 N-sodium hydroxide. Chromatography of the ether extract in solvent (b) gave one spot, R_F 0.85, possessing the following properties: it gave a yellowish-green fluorescence in ultraviolet light, a 2,4-dinitrophenylhydrazone derivative, a positive *o*-dianisidine test for an aldehyde, a positive Folin test for phenols and a colour with diazotized *p*-nitroaniline reagent. In N-hydrochloric acid the absorption $\lambda_{\max}$ were at 257, 297 and 375 m μ ; in N-sodium hydroxide it gave a yellow colour with $\lambda_{\max}$ at 267, 320 and 405 m μ . From the remainder of the ether extract the 2,4-dinitrophenylhydrazone was

prepared in the usual manner, and the bright-orange solid recrystallized from benzene, m.p. 299–301° (decomp.) undepressed by an authentic sample of 1-hydroxy-2-naphthaldehyde-2,4-dinitrophenyl-hydrazone. The infrared spectra of the two derivatives were also identical.

In a similar manner, this crude cell-free extract (fraction A) gave 1-hydroxy-2-naphthaldehyde when allowed to act on the ring-fission product, *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid. The enzyme involved in this reaction appeared to be very stable; dialysis against running tap water for 3 days caused no loss of activity and it was insensitive to potassium cyanide (1 mM).

Conversion of 1-hydroxy-2-naphthaldehyde into 1-hydroxy-2-naphthoic acid by a dehydrogenase. The oxidation of 1-hydroxy-2-naphthaldehyde to the acid by the crude extract (fraction A) specifically required the addition of NAD; NADP was ineffective. Manometrically, the reaction was shown to involve the consumption of 1 atom of oxygen/mol. of substrate. The product was identified by chromatography in solvent (b), R_F 0.75, giving a dark-blue fluorescence under ultraviolet light, a purple-brown colour with the diazotized *p*-nitroaniline reagent and ultraviolet spectrum of the eluted spot ($\lambda_{\max}$ 247 m μ). This enzyme system was also capable of oxidizing 2-hydroxy-3-naphthaldehyde and salicylaldehyde to 2-hydroxy-3-naphthoic acid and salicylic acid respectively. With NAD in the crude cell-free extract, it oxidized 3,4-dihydroxyphenanthrene all the way to 1-hydroxy-2-naphthoic acid.

Further metabolism of 1-hydroxy-2-naphthoic acid. Phenanthrene-grown cell suspensions metabolized 1-hydroxy-2-naphthoic acid, 1,2-dihydroxynaphthalene and catechol immediately. If cells that had been stored at –15° for several weeks were used, the reaction mixture developed a yellow colour, extractable into ether at acid pH and becoming colourless. Its ultraviolet-absorption spectrum with $\lambda_{\max}$ at 317 m μ changing to 373 m μ in alkali, a positive *o*-dianisidine test for aldehyde and acidic 2,4-dinitrophenylhydrazone showed it to be α -hydroxymuconic semialdehyde (Dagley, Evans & Ribbons, 1960). Incubation of catechol with the standard purified enzyme (fraction B) also gave this compound; with 1,2-dihydroxynaphthalene as substrate, the product was *cis*-*o*-hydroxybenzalpyruvate (Davies & Evans, 1964), as shown by its ultraviolet absorption $\lambda_{\max}$ at 257 and 298 m μ in acid, changing to 306 and 420 m μ and becoming yellow in alkali, and on chromatography in solvents (a) and (c) one spot with R_F 0.95 and 0.98 respectively, corresponding to this compound. It therefore appears probable that 1-hydroxy-2-naphthoate is oxidatively decarboxylated to 1,2-dihydroxynaphthalene, although this has not yet been demonstrated with a cell-free system.

Anthracene metabolism

All the organisms used in the present investigation grew very slowly in anthracene–mineral salts media, even with homogenization of the substrate and with the most favourable shake-culture conditions. Crop yields were therefore low, and this temporarily hampered further progress. It was found, however, that if naphthalene-grown cells were washed aseptically in phosphate buffer, pH 7 (0.05 M), re-suspended in sterile anthracene–mineral salts medium and aerated, adaptation to the new substrate occurred within 24 hr., since one of the known intermediates, 2-hydroxy-3-naphthoic acid, could be detected in culture, by chromatography in solvent (a), R_F 0.15, and characteristic absorption $\lambda_{\max}$ (at 236 m μ), after acid–ether extraction.

Sequential-induction experiments. With anthracene-grown cells (strain P_G) harvested as young as possible, or naphthalene-grown organisms readapted to anthracene, the results of Rogoff & Wender (1957b) were confirmed, i.e. oxidation of 2-hydroxy-3-naphthoate, salicylate and catechol occurred immediately. In addition, there was strong induction to low concentrations of 1,2-dihydroxyanthracene and 2-hydroxy-3-naphthaldehyde (Table 2). 2,3-Dihydroxynaphthalene and 6,7-benzocoumarin gave oxygen uptakes above the control value, but the test was not convincing in their case.

It was subsequently found that naphthalene-grown cells, even without readaptation to anthracene, actively metabolized 1,2-dihydroxyanthracene and 2-hydroxy-3-naphthaldehyde. Again, a similar induction pattern to that shown by anthracene-adapted cells was obtained with 2,3-dihydroxynaphthalene and 6,7-benzocoumarin. Even anthracene itself was metabolized at a reasonable rate by naphthalene-grown cells (cf. Rogoff, 1962). Since it was possible to produce large quantities of naph-

Table 2. *Oxygen consumption by anthracene-adapted cells with different substrates*

Each Warburg flask contained cell suspension (0.5 ml.; approx. 15 mg. dry wt./ml.) and substrate [2 μ moles of (A) in 0.5 ml. added from the side arm, or 1.5 μ moles of (B) that had been precipitated on to a small piece of filter paper from anhydrous acetone solution]. The solutions above were made up in Na₂HPO₄–KH₂PO₄ buffer, pH 6.5 (0.05 M), which was also used to make up the total volume to 3 ml. The centre well contained 0.2 ml. of 4 N-KOH.

Substrate	O ₂ taken up in first 10 min. (μ l.)
No added substrate	6
(A) 6,7-Benzocoumarin	8
(A) 2-Hydroxy-3-naphthaldehyde	28
(B) 1,2-Dihydroxyanthracene	49

thalene-grown cells, which contained the enzymes capable of dissimilating 1,2-dihydroxyanthracene, these were used in all the subsequent enzymic experiments.

Oxidation of 1,2-dihydroxyanthracene by cell-free extracts. Dilution of the cell-free extract obtained from naphthalene-grown cells (18 mg. of protein/ml.) caused the reaction to stop after the ring-fission step. The cell-free systems from phenanthrene-grown cells (fractions A and B) described above also metabolized 1,2-dihydroxyanthracene: again mere dilution of the crude cell-free extract caused the reaction to stop after the ring-fission step. 1,2-Dihydroxyanthracene, like 1,2-dihydroxynaphthalene (Davies & Evans, 1964), is unstable in aqueous solutions above pH 7; to minimize the formation of 1,2-anthraquinone, reactions were carried out at pH 6.5 and enzyme solution of sufficient activity to complete the cleavage of 1,2-dihydroxyanthracene within a few minutes was employed. An approximate estimation of the activity of these extracts was obtained as follows. Serially diluted cell-free extract (18 mg. of protein/ml.) in a total volume of 3 ml. of disodium hydrogen phosphate-potassium dihydrogen phosphate buffer, pH 6.5 (0.05 M), was incubated with 1,2-dihydroxyanthracene (1 μ mole) that had been precipitated on to a small piece of filter paper from an anhydrous acetone solution. The greatest dilution that was still capable of preventing the formation of the orange-brown 1,2-anthraquinone was observed. By this method it was consistently found that Fe^{2+} (1 mM) increased enzyme activity; also, that the enzyme was sensitive to oxygen and could be reactivated by the sodium borohydride treatment (see above). Heating at 50° for 5 min. in the pH range 5–8 resulted in complete inactivation.

The cell-free extract (18 mg. of protein/ml.) suitably diluted (0.1 ml.) in phosphate buffer, pH 6.5 (0.05 M), containing ferrous sulphate (1 mM), to a total volume of 3 ml. metabolized 1 μ mole of 1,2-dihydroxyanthracene/ml. One mol. of oxygen was consumed/mol. of substrate but no carbon dioxide was evolved; the automatic titrator, in a parallel experiment, showed that 1 carboxyl group was produced/mol. of substrate.

In a large-scale enzymic oxidation of 1,2-dihydroxyanthracene, with the relative quantities of reactants described above, the ring-fission product was extracted into ether at acid pH, and from the ether into 4% sodium hydrogen carbonate solution. Reacidification of this, followed by ether extraction and careful evaporation of most of the ether under reduced pressure, gave a concentrate that on chromatography in solvent (a) gave one spot, R_F 0.47, possessing the following properties: (i) it had a deep-purple fluorescence under ultraviolet light; (ii) it gave a faint brown colour when exposed

to ammonia fumes; (iii) it gave a positive reaction with 2,4-dinitrophenylhydrazine after some time, showing the presence of a keto group; (iv) it gave a positive Folin-Ciocalteu reaction for a phenol group; (v) it gave a reddish colour with diazotized *p*-nitroaniline-sodium carbonate reagent.

In 0.1 N-hydrochloric acid the solution was colourless, with ultraviolet absorption λ_{max} at 247, 290 and 302 μ ; in 0.1 N-sodium hydroxide it gradually went yellow after a few hours at room temperature with absorption λ_{max} at 316 and 435 μ (Fig. 2).

2,4-Dinitrophenylhydrazone of the ring-fission product. Three 10 mg. quantities of 1,2-dihydroxyanthracene were added with stirring at 10 min. intervals to 10 ml. of the standard enzyme solution (18 mg. of protein/ml.) in 300 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, 10 min. after the final addition of substrate, the reaction mixture was extracted with ether (5 $\times$ 100 ml.) to remove any neutral components. The aqueous phase was then acidified to Congo red with dilute sulphuric acid and re-extracted with ether (5 $\times$ 100 ml.) to remove the ring-fission product. The combined ether extract was dried with anhydrous sodium sulphate, filtered and solid anhydrous barium chloride added to remove traces of sulphate. The filtered ether extract was concentrated to 30 ml. under reduced pressure, and shaken with 20 ml. of 0.1 N-sodium hydroxide. The alkaline layer was separated from the ether, acidified with dilute hydrochloric acid and treated with a slight excess of 2,4-dinitrophenylhydrazine in 2 N-hydrochloric acid. The reaction mixture was allowed to stand at 25° for 6 hr., and then overnight in the cold room.

Fig. 2. Ultraviolet-absorption spectra in 0.1 N-NaOH (—) and in 0.1 N-HCl (---) of the ring-fission product of 1,2-dihydroxyanthracene.

The orange-red solid was collected, washed with 2*N*-hydrochloric acid and then water, and dried *in vacuo*. Recrystallization from benzene gave a microcrystalline powder, m.p. 109–112° (Found: N, 13.3. $C_{20}H_{14}N_4O_7$ requires N, 13.3%).

Conversion of the ring-fission product of 1,2-dihydroxyanthracene into 6,7-benzocoumarin. Two 10mg. quantities of 1,2-dihydroxyanthracene were added with stirring at 10min. intervals to 10ml. of the standard enzyme solution (18mg. of protein/ml.) in 300ml. of disodium hydrogen phosphate–potassium dihydrogen phosphate buffer, pH 6.5 (0.05*M*), containing ferrous sulphate (1*mM*), and incubated at 30°. Then, 10min. after the final addition of substrate, the reaction mixture was extracted with ether (5 × 100ml.) to remove any neutral components. The aqueous phase was acidified to Congo red with dilute sulphuric acid and re-extracted with ether (5 × 100ml.) to remove the ring-fission product. The combined ether extract was dried with anhydrous sodium sulphate, the drying agent removed by filtration and solid anhydrous barium chloride added to the ether to remove traces of sulphate. The filtered ether extract was concentrated under reduced pressure to 15ml. and shaken with 10ml. of 0.1*N*-sodium hydroxide. The aqueous alkaline layer was separated from the ether, and contained the sodium salt of the ring-fission product, as shown by the ultraviolet-absorption spectrum and chromatography. It was treated with 0.2ml. of 30% (w/v) hydrogen peroxide and kept at room temperature for 40min. The solution was then acidified with dilute sulphuric acid, left for 30min. and extracted with chloroform (3 × 10ml.); the combined chloroform extract was washed with 4% sodium hydrogen carbonate and then water, and finally dried with anhydrous sodium sulphate. Examination of the chloroform extract showed it to have ultraviolet-absorption λ_{max} at 267, 278, 317 and 326*mμ*. Chromatography in solvent (c) showed one spot, R_F 0.87, and a greenish-blue fluorescence under the Chromatolite lamp; it gave an orange colour with the diazotized *p*-nitroaniline reagent after some time. Evaporation of the chloroform under reduced pressure left a solid that after recrystallization from benzene had m.p. 160–163° undepressed by an authentic sample of 6,7-benzocoumarin.

Structure of the ring-fission product of 1,2-dihydroxyanthracene: *cis*-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid. Attempts to isolate the ring-fission product from an ethereal solution resulted in decomposition. Its ready conversion into 6,7-benzocoumarin, the presence of one carboxyl radical, a carbonyl group and phenolic group, together with the gaseous exchange during its formation, are consistent with the view that fission has occurred between the angular C and C-1 giving *cis*-4-(2-hyd-

roxynaphth-3-yl)-2-oxobut-3-enoic acid. Analysis of the 2,4-dinitrophenylhydrazone derivative supports this; ultraviolet-absorption spectrum characteristics in acid and alkali are also those to be expected from such a compound. The infrared spectrum is discussed below.

Condensation of 2-hydroxy-3-naphthaldehyde with pyruvic acid did not take place, either in methanolic potassium hydroxide or in ether and dry hydrochloric acid. 2-Hydroxy-3-naphthoic acid was the only product isolated.

Action of crude cell-free extracts on 1,2-dihydroxyanthracene. When crude undiluted cell-free extract was incubated with 1,2-dihydroxyanthracene in the presence of Fe^{2+} (1*mM*) and phosphate buffer, pH 6.5 (0.5*M*), a different product was obtained to that already described. The reaction mixture was separated into a neutral-ether- and an acidic-ether-soluble component; the latter had R_F 0.15 on chromatography in solvent (a). Its yellowish-green fluorescence and ultraviolet-absorption spectrum showed it to be 2-hydroxy-3-naphthoic acid. The neutral-ether fraction had R_F 0.85 on chromatography in solvent (b), a positive aldehyde and Folin-Ciocalteu reaction, a yellow fluorescence and absorption spectrum corresponding to 2-hydroxy-3-naphthaldehyde.

When *cis*-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid was substituted for 1,2-dihydroxyanthracene in this enzymic action, together with NAD, the product was 2-hydroxy-3-naphthoic acid.

Conversion of 2-hydroxy-3-naphthaldehyde into 2-hydroxy-3-naphthoic acid by a dehydrogenase present in the cell-free extract. The oxidation of 2-hydroxy-3-naphthaldehyde by a cell-free system specifically required the addition of NAD; NADP was inactive. The presence of a cytochrome system enabled the reaction to be followed manometrically; it was shown to involve the consumption of 1 atom of oxygen/mol. of substrate. The product, 2-hydroxy-3-naphthoic acid, was isolated and characterized.

This cell-free system from naphthalene-grown cells also oxidized 1-hydroxy-2-naphthaldehyde and salicylaldehyde to the corresponding acids under similar conditions; it therefore appears to be due to one dehydrogenase, of low specificity.

Further metabolism of 2-hydroxy-3-naphthoic acid. From anthracene-grown cultures Fernley (1959) had already shown the presence of 2,3-dihydroxynaphthalene in the following manner. Shake cultures of *Pseudomonas aeruginosa* (Rogoff) developed an increasingly positive Folin reaction after several days' growth. One such culture (250ml.) aged for 16 days was harvested, and the supernatant acidified and extracted with benzene. Partition of the components of this extract into neutral-ether- and acidic-ether-soluble fractions showed the presence of 2-hydroxy-3-naphthoic acid in the latter (cf.

Rogoff & Wender, 1957b). The neutral-ether fraction gave a strong reaction for an *o*-dihydroxyphenol (Evans, 1947), a deep-blue colour with ferric chloride, and chromatography in solvents (c) and (d) gave R_f 0.72 and 0.78 respectively; the ultraviolet absorption $\lambda_{\max}$ was at 228 μ . Evaporation of the solvent left a few milligrams of an off-white solid, recrystallized from water, m.p. 160° undepressed by an authentic sample of 2,3-dihydroxynaphthalene.

It is surmised that 2-hydroxy-3-naphthoic acid is oxidatively decarboxylated to 2,3-dihydroxynaphthalene, but no cell-free system has yet been obtained that metabolized either of these intermediates. In conformity with the observation of Rogoff & Wender (1957b) that catechol is a member of this pathway, it was easily demonstrated that a cell-free system of anthracene-grown bacteria acted on catechol to give α -hydroxymuconic semialdehyde.

Infrared spectra of the keto acids produced by enzymic cleavage of 1,2-dihydroxynaphthalene 3,4-dihydroxyphenanthrene and 1,2-dihydroxyanthracene. The keto acids resulting from fission of these *o*-dihydroxyphenol derivatives at the bond between the angular C and that bearing the hydroxyl group should all be derivatives of the known benzalpyruvic acid. A comparison of their infrared spectra would therefore be expected to provide valuable additional evidence about the structures proposed for them. Accordingly, the potassium salts were prepared by the following general method.

Purified enzyme (fraction B) was allowed to attack the appropriate *o*-dihydroxyphenol substrate under the conditions described above. The acidic fission product was extracted into ether, then taken into potassium hydrogen carbonate solution, followed by acidification and re-extraction into ether. This ethereal solution was dried with anhydrous sodium sulphate, filtered, treated with anhydrous barium chloride to remove traces of the sulphate, again filtered and finally concentrated to a small volume under reduced pressure. The ether concentrate was carefully neutralized with the calculated amount of potassium hydroxide in methanol to pH 7; at this point a cloudiness appeared. More dry ether was added, and the potassium salt that was precipitated was allowed to settle. The supernatant was decanted, and the powdery solid washed with dry ether and kept *in vacuo* over silica gel. Each preparation was checked for purity by chromatography and ultraviolet absorption before incorporation into the potassium chloride disk. Potassium benzalpyruvate synthesized by the method of Reimer (1931) and Friedmann (1931) was coloured yellow, potassium *o*-hydroxybenzalpyruvate (Davies & Evans, 1964) was yellowish-orange, potassium *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoate was orange-brown and potassium *cis*-4-

(2-hydroxynaphth-3-yl)-2-oxobut-3-enoate was yellow.

Infrared spectrum of potassium cis-o-hydroxybenzalpyruvate. The infrared spectrum (Fig. 3) is consistent with the hemiacetal form of structure assigned to this compound. A strong absorption band found at 3350 cm^{-1} is due to a hydroxyl group, which, as may be expected, is absent from the infrared spectrum of potassium benzalpyruvate.

The absence of a carbonyl peak is explained by its involvement with the phenolic group, the salt being in the hemiacetal form. In potassium benzalpyruvate, where hemiacetal formation is not possible, a sharp peak occurs in the spectrum at 1680 cm^{-1} ; this is attributed to the carbonyl group that has been influenced by an $\alpha\beta$ -unsaturated bond, known to lower the frequency. Two absorption bands between 1000 and 1100 cm^{-1} may be attributed to the stretching vibrations of an ether linkage. As with potassium benzalpyruvate the antisymmetrical and symmetrical vibrations of the carboxylate ion show up as bands at 1610 and 1390 cm^{-1} . Absorption at 755 cm^{-1} is in the region (770–735 cm^{-1}) where bands due to *ortho*-disubstituted benzene derivatives occur.

Infrared spectrum of potassium cis-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoate. Analysis of the infrared spectrum of this compound (Fig. 4) is con-

Fig. 3. Infrared spectrum of potassium *cis*-*o*-hydroxybenzalpyruvate.

Fig. 4. Infrared spectrum of potassium *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoate.

sistent with the view that it is the potassium salt of *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid in the hemiacetal form. The strong absorption peak of a hydroxyl group appears at 3350 cm^{-1} . Again, the absence of a carbonyl peak is attributed to its involvement in hemiacetal formation. The absorption at 1100 cm^{-1} is due to the stretching vibrations of an ether linkage. Symmetrical and antisymmetrical vibrations of the carboxylate ion show up as characteristic peaks at 1390 and 1620 cm^{-1} . According to Flett (1963) a 1,2-disubstituted naphthalene nucleus has absorption peaks at about 734 , 810 , 940 , 1151 , and 1211 cm^{-1} ; although no definite assignments can be made, the compound does show peaks in these regions, e.g. at 730 cm^{-1} , a doublet at 795 and 800 cm^{-1} , 935 , 1160 and 1215 cm^{-1} . No absorption is evident at 990 cm^{-1} , where *trans*-ethylenic linkages are known to absorb quite strongly. This is consistent with a hemiacetal structure that demands a *cis*-configuration; it is possible that the absorption at 795 cm^{-1} is due to the out-of-plane hydrogen deformations in an ethylenic bond.

Infrared spectrum of potassium cis-4-(2-hydroxynaphth-2-yl)-2-oxobut-3-enoate. As expected (Fig. 5), some similarity with the infrared spectrum of potassium *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoate is evident. Bands due to hydroxyl and carboxylate ion were present as above. Absence of a carbonyl absorption is again explained by hemiacetal formation. A 2,3-disubstituted naphthalene nucleus has absorption bands at 824 , 1160 , 1193 and 1263 cm^{-1} (Flett, 1963). Although no definite assignments can be made, the molecule in question does show peaks at 832 , 1160 , 1205 (shoulder) and 1250 cm^{-1} . No strong absorption bands are found in the $990\text{--}965\text{ cm}^{-1}$ region, where a *trans*-ethylenic group, if present, would absorb. Because of the uncertainty in position of *cis*-ethylenic absorptions, no profitable correlations can be made, although bands exist in this spectrum at 705 and 750 cm^{-1} , i.e. where they have been found to occur.

Fig. 5. Infrared spectrum of potassium *cis*-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoate.

DISCUSSION

Scheme 1 illustrates the proposed pathway of phenanthrene (I) dissimilation by soil pseudomonads. Perhydroxylation produces *trans*-3,4-dihydro-3,4-dihydroxyphenanthrene (II) (Colla *et al.* 1959), which is then dehydrogenated to 3,4-dihydroxyphenanthrene (III). Neither of these steps has been demonstrated enzymically. Ring-cleavage of compound (III) between the angular C and C-4 takes place through the action of an Fe^{2+} -dependent oxygenase, which was extracted from the microorganisms. The fission product, *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid (IV), is itself unstable [being readily decarboxylated and oxidized to 7,8-benzocoumarin (VII)], but afforded a crystalline 2,4-dinitrophenylhydrazine derivative. It exists in various tautomeric forms, depending on the pH of the aqueous solution; in neutral or slightly acid media it assumes the almost colourless hemiacetal form (V); in strong acid it becomes coloured, with the probable formation of a pyrylium cation (VI), although a crystalline perchlorate could not be prepared in this case (cf. *cis*-*o*-hydroxybenzalpyruvic perchlorate; Davies & Evans, 1964). In alkali, the coloured anion of compound (IV) is formed. An enzyme of the aldolase type attacks the side chain to give 1-hydroxy-2-naphthaldehyde (IX), possibly via the intermediate (VIII); the fate of the C_3 fragment has not yet been ascertained, but it could well be initially eliminated as pyruvate. An NAD-dependent dehydrogenase then converts the aldehyde into 1-hydroxy-2-naphthoic acid (X). Since 1,2-dihydroxynaphthalene (XI) satisfies all the criteria of an intermediate, it is thought to originate by oxidative decarboxylation of compound (X), although a cell-free system has not yet been obtained to reconstitute this step. Its formation allows continuation of metabolism via the naphthalene pathway (Davies & Evans, 1964).

Scheme 2 depicts the pathway suggested for the metabolism of anthracene (XII). The initial production of *trans*-1,2-dihydro-1,2-dihydroxyanthracene (XIII) rests on the evidence of Colla *et al.* (1959); neither the perhydroxylation nor the dehydrogenation reaction to give 1,2-dihydroxyanthracene (XIV) has yet been demonstrated enzymically. An Fe^{2+} -dependent oxygenase disrupts compound (XIV) at the bond between the angular C and C-1, giving *cis*-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid (XV). The fission product shows properties analogous to those of compound (IV) and *cis*-*o*-hydroxybenzalpyruvate, namely its ready conversion into 6,7-benzocoumarin (XVIII) and its existence in various tautomeric forms, the colourless hemiacetal (XVI) in neutral and weakly acid solution, a coloured pyrylium cation (XVII) in strong acid, and a coloured anion (XV) in alkali. Attempts

Scheme 1. Proposed pathway of phenanthrene metabolism by soil pseudomonads.

to isolate compound (XV) in crystalline form led to decomposition, but it gave a crystalline 2,4-dinitrophenylhydrazine derivative. Attack on the side chain of the keto acid to give 2-hydroxy-3-naphthalaldehyde (XX) was demonstrated with a cell-free enzyme system; the addition of NAD allowed a dehydrogenase, which was also present, to oxidize this to 2-hydroxy-3-naphthoic acid (XXI). The origin of 2,3-dihydroxynaphthalene (XXII), which was isolated from anthracene-adapted cultures, is

thought to be by oxidative decarboxylation of the acid (XXI); it is not known precisely how compound (XXII) is metabolized through to salicylate. The ring-fission enzyme, prepared from cells grown on naphthalene, anthracene, phenanthrene or even benzoate, oxidatively disrupts 1,2-dihydroxynaphthalene (Davies & Evans, 1964), 3,4-dihydroxyphenanthrene and 1,2-dihydroxyanthracene. Cleavage takes place at the bond between the angular C and that bearing the hydroxyl group in every case, not,

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

as Rogoff (1962) assumed, at the bond between the two hydroxyl groups. Cleavage of catechol with these cell-free preparations is always at the bond between C-2 and C-3. This is the action of catechol 2,3-oxygenase (Dagley *et al.* 1960), and it appears probable that all these ring-fission steps are performed by one and the same enzyme, although rigorous proof will have to await the preparation of the pure enzyme. With *Pseudomonas* strains P_F and P_G it was found that this enzyme is constitutive in these organisms, since it could be extracted from the cells irrespective of the substrate used for growth, e.g. glucose, succinate, glycerol and benzoate, in addition to the polycyclic aromatic hydrocarbons.

Rogoff (1962) suggests that the bacterial enzyme systems induced to deal with the angular hydrocarbon phenanthrene differ from those evolved to cope with the linearly arranged naphthalene and anthracene. In the latter hydrocarbons, the C-C bonds of high electron density ('K regions'; Pullman & Pullman, 1955) are localized in the 1,2-

position: attachment to an enzyme and ring-splitting may take place at the same ring in both cases; hence the enzymes are similar. Phenanthrene has its 'K region' at the 9,10-bond: if attachment to an enzyme takes place at this site, while the ring fission is occurring in another ring, the two-ring span of this enzyme would make it very different from that in the other two cases. It now appears that the oxidative cleavage step is catalysed by a common enzyme of relatively low specificity. Rogoff's (1962) conclusions therefore do not apply to this stage of metabolism. Whether they are true for the initial perhydroxylating or dehydrogenating reactions will have to await the preparation of the relevant cell-free enzymes.

Soils, even from rural areas, have been shown to contain a large variety of polynuclear aromatic hydrocarbons, e.g. 3,4-benzopyrene, 1,2-benzopyrene, chrysene, phenanthrene, anthracene, 1,12-benzoperylene, fluoranthrene, pyrene, perylene, anthanthrene, triphenylene, benzanthracene, benzofluorene and coronene (Kern, 1947; Blumer, 1961).

The latter author ascribes their origin to plant organic matter and regards them as indigenous to soil. Several of these hydrocarbons also occur in urban airborne particulate matter (Sawicki, Elbert, Stanley, Hauser & Fox, 1960).

Interest in the biochemistry of polynuclear aromatic hydrocarbons stems from the fact that some are carcinogens; their metabolism in mammals has been extensively studied (Parke & Williams, 1959). It now transpires that similarities exist between the initial mode of attack by bacterial and liver enzymes, e.g. dihydroarenediol formation is a common phenomenon. Although in animals these polynuclear hydrocarbons are excreted in the form of various conjugated derivatives, soil pseudomonads can completely dissimilate them.

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