The Metabolism of Aromatic Compounds by *Rhodopseudomonas palustris*

A NEW, REDUCTIVE, METHOD OF AROMATIC RING METABOLISM

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1. *Rhodopseudomonas palustris* grows both aerobically and photosynthetically on aromatic acids. *p*-Hydroxybenzoate and protocatechuate are able to support aerobic growth; these compounds are metabolized by the protocatechuate 4,5-oxygenase pathway. 2. The photoassimilation of benzoate and hydroxybenzoates and the effects of air and darkness on the photoassimilation of benzoate are described. 3. Evidence in conflict with the pathway previously proposed for the photometabolism of benzoate is discussed. 4. The photometabolism of benzoate is accomplished by a novel reductive pathway involving its reduction to cyclohex-1-ene-1-carboxylate, followed by hydration to 2-hydroxycyclohexanecarboxylate and after dehydrogenation to 2-oxocyclohexanecarboxylate further hydration results in ring-fission and the production of pimelate. 5. Attempts were made to prepare cell-free extracts capable of dissimilating benzoate.

From extensive studies on the oxidative metabolism of aromatic compounds by aerobic micro-organisms, many of the pathways and mechanisms involved have been described (Evans, 1963; Ribbons, 1965; Dagley, 1967). Bacteria that dissimilate aromatic compounds under anaerobic conditions have also been reported. Tarvin & Buswell (1934) and Barker (1956) described several aromatic acids that were utilized under strictly anaerobic conditions by the methanogenic bacteria; Harary (1956) isolated a *Clostridium* sp. capable of anaerobic fermentation of nicotinate; and Scher & Proctor (1960) isolated several strains of non-sulphur photosynthetic bacteria that utilized benzoate anaerobically in the light.

Clark & Fina (1952) showed that cultures of methanogenic bacteria grown on benzoate failed to metabolize catechol or protocatechuate, and Roberts (1962) reported that similar benzoate-induced cultures produced methane without a lag when incubated with cyclohexanecarboxylate, butyrate or propionate, whereas long induction periods were required with fumarate, acrylate or 2-methylpropionate, which suggested a method of aromatic metabolism different from the known aerobic type and one that possibly is reductive. The

first stage of the anaerobic fermentation of nicotinate by a *Clostridium* sp. has been shown to involve hydration to form 6-hydroxynicotinate (Harary, 1957*a,b*), and several of the later intermediates have been described (Tsai, Pastan & Stadtman, 1966). In studies on the photometabolism of benzoate by a *Rhodopseudomonas* sp. Proctor & Scher (1960) proposed a pathway that is typically oxidative, which invoked protocatechuate and catechol as intermediates. The absence of enzymes of these oxidative pathways from *R. palustris* grown photosynthetically on aromatic substrates has, however, been demonstrated (Hegeman, 1967; Dutton & Evans, 1967), and the present paper provides further evidence by describing a new reductive route for the metabolism of the benzene ring. Preliminary accounts of this work have been published (Dutton & Evans, 1967, 1968*a,b*).

MATERIALS AND METHODS

Culture techniques

Rhodopseudomonas palustris (Scher strain), a gift from Dr J. Turner (Department of Biochemistry, University of Liverpool), was obtained as a *Rhodopseudomonas* sp. but identified from its microscopic appearance (van Niel, 1944) and requirement for *p*-aminobenzoate (Hutner, 1950) as *Rhodopseudomonas palustris*. It was grown photosynthetically under O₂-free N₂ or aerobically in normal laboratory light conditions; in either case the following medium was

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used: organic substrate, 0.5 g./l. (aromatic acids) or 1.5 g./l. (aliphatic acids); $(\text{NH}_4)_2\text{SO}_4$, 1.0 g./l.; K_2HPO_4 , 0.5 g./l.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g./l.; $\text{FeSO}_4 \cdot 5\text{H}_2\text{O}$, 25 mg./l.; *p*-aminobenzoate, 0.1 mg./l.; biotin, 0.05 mg./l.; thiamin hydrochloride, 1.0 mg./l.; nicotinic acid, 1.0 mg./l. The medium was made up with tap water, adjusted to pH 7.2–7.4 with NaOH and autoclaved for 20 min. at 20 lb./in.².

The organism was grown photosynthetically at 30–32° in 5 l. Pyrex bottles in a glass-sided tank illuminated on both sides by a bank of eight to ten 60 w tungsten lamps. After 2 days' growth on media containing benzoate a further 1.5 g. of sodium benzoate was usually added, the pH of the culture being adjusted at the same time with NaOH to pH 7.2; the cells were generally harvested after 65–72 hr. The culture, maintained in a benzoate–mineral salts medium, was transferred, by using a 5% inoculum, every 3–4 days. Cultures grown with hydroxybenzoates as substrates were started with 250 ml. of inoculum from a culture grown photosynthetically on acetate. The cells were harvested by centrifugation at 5000g for 5 min. at 0° in an MSE High Speed 18 centrifuge and the pellet was washed once with about 10 vol. of 0.05 M-tris-HCl or 0.05 M-phosphate (K_2HPO_4 -NaOH) buffer, pH 7.2, centrifuged again and used immediately.

For aerobic growth, baffled flasks (1 l.) containing 250 ml. of medium were inoculated from a slope and after 3–4 days in a rotary shaker (Gallenkamp Orbital Shaker) (130 strokes/min.) the contents were transferred to a 5 l. or 10 l. culture vessel. After 3–4 days' forced aeration at 30°, towards the end of the exponential growth phase, the cells were harvested in the Sharples Super centrifuge, washed once with 0.05 M-phosphate buffer, pH 7.2, and stored at –20°. The aerobically growing cultures were not kept in the dark; Lascelles (1956) has noted that normal laboratory light conditions have little or no effect on photosynthetic bacteria growing aerobically.

Analytical techniques

Spectrophotometric determinations were carried out in the Beckman DB or Unicam SP. 800 recording spectrophotometers.

Benzoic acid and the phenolic acids in culture media were determined as follows: after centrifugation, the clear supernatant (5.0 ml.) was acidified with 0.2 ml. of 1.5 M- H_2SO_4 and extracted twice with 10 ml. of ether. The combined extracts were washed with 5 ml. of distilled water (acidified to pH < 3) and the aromatic acid was extracted from the ether into 5.0 ml. of 4% (w/v) NaHCO_3 ; the extinction was measured at 270 nm. for benzoate and at suitable wavelengths for the phenolic acids. Phenolic compounds were also determined with the Folin–Ciocalteu reagent.

Bacterial dry weight was determined from the extinction at 660 nm., by reference to a standard calibration curve.

Protein was determined as described by Warburg & Christian (1942).

Manometric experiments were conducted in the conventional Warburg constant-volume apparatus at 30°; O_2 uptake was measured by the direct method (Dixon, 1952).

Radioactive materials that were coloured or insoluble were assayed as a solid film of standard thickness on aluminium planchets by using a Geiger–Müller tube in conjunction with an I.D.L. scaler. Soluble radioactive

compounds were assayed in an NE 8304 automatic liquid-scintillation spectrometer (Nuclear Enterprises Ltd., Edinburgh). The scintillation fluids employed were NE 213 and NE 220 (Nuclear Enterprises Ltd.). The maximum efficiency for counting ^{14}C was approx. 90%, corrections for quenching being made when necessary.

Values for melting points are uncorrected.

Reagents

Chemicals. Benzoic acid, sodium benzoate, the mono-hydroxybenzoic acids, 3,4- and 2,6-dihydroxybenzoic acid and catechol were obtained from British Drug Houses Ltd., Poole, Dorset; the other dihydroxybenzoic acids (except 2,3-dihydroxybenzoic acid, which was prepared by the method of Perkin & Robinson, 1914), pimelic acid, cyclohexanecarboxylic acid and 2,4-lutidinic acid came from Koch–Light Laboratories Ltd., Colnbrook, Bucks.; 2-oxo-pimelic acid came from K & K Laboratories Inc., Plainview, N.Y., U.S.A., and cyclohex-3-ene-1-carboxylic acid from Ralph N. Emanuel Ltd., London. Analytical-grade reagents were used where available.

In the preparation of cyclohex-1-ene-1-carboxylic acid, the cyclohexanone cyanohydrin, prepared by the method of Ruzicka & Brugger (1926), was converted into cyclohexene-1-carbonitrile by the method of Chakravarti (1947). This was hydrolysed by refluxing for 3½ hr. with 10 vol. of 18 M- H_2SO_4 -acetic acid–water (1:1:1, by vol.) (Babor & Bloch, 1966). The product was recrystallized from light petroleum (b.p. 40–60°) and had m.p. 36–38° (reported m.p. 36–37°).

Cyclohex-2-ene-1-carboxylic acid was prepared as described by Boorman & Linstead (1935) and purified by debromination of the crystallized 2,3-dibromocyclohexanecarboxylic acid, which had m.p. 167–169° (reported m.p. 167°).

The preparation of 2-oxocyclohexanecarboxylic acid (m.p. 75–76°, decomp.; reported m.p. 79–81°, decomp.) and its reduction to 2-hydroxycyclohexanecarboxylic acid (m.p. 109–110°; reported m.p. 111°) were as described by Gardner, Perkin & Watson (1910).

trans-4-Hydroxycyclohexanecarboxylic acid was a gift from Dr F. Dickens, Dr N. R. Campbell and J. H. Hunt.

Radiochemicals. [*ring*- ^{14}C]Benzoic acid (specific radioactivity 395 $\mu\text{C}/\text{mg.}$) and [*carboxy*- ^{14}C]benzoic acid (specific radioactivity 160 $\mu\text{C}/\text{mg.}$) were obtained from The Radiochemical Centre, Amersham, Bucks. These were purified by two-dimensional t.l.c. on silica gel G developed by solvents (a) and (c) (for short-exposure experiments), or solvent (e) in both directions (for isotope-dilution experiments). The benzoic acid spot was located by radioautography, closely cut out, eluted and taken up into a few millilitres of 0.05 M-tris-HCl buffer, pH 7.2, and used, undiluted, immediately or on the following day.

Solvents. Solvents used for extraction or preparative purposes were generally redistilled. Ether was redistilled over 'reduced iron'; ethanol was refluxed for 6 hr. over NaOH pellets (40 g./l.) and Zn dust (20 g./l.) and then distilled.

Chromatography

Analytical chromatography was performed by t.l.c. according to the methods of Randerath (1963). The glass plates (20 cm. × 20 cm.) were coated to a thickness of

0.25 mm. and the following systems were used. With silica gel G (E. Merck A.-G., Darmstadt, Germany): (a) benzene-methanol-acetic acid (45:8:4, by vol.) and (b) (45:8:2, by vol.); (c) toluene-ethyl acetate-formic acid (5:4:1, by vol.) and (d) (15:6:1, by vol.); (e) benzene-dioxan-acetic acid (45:5:2, by vol.); (f) light petroleum (b.p. 40–60°)-diethyl ether-acetic acid (50:50:1, by vol.) and (g) (80:20:1, by vol.); (h) light petroleum (b.p. 40–60°)-diethyl ether (9:1, v/v); (i) methanol-chloroform-water (20:75:3, by vol.) and (j) (25:65:4, by vol.); (k) ethanol-aq. NH_3 (sp.gr. 0.88)-water (25:1:6, by vol.) and (l) (16:1:3, by vol.); (m) butan-1-ol-ethanol-water (7:1:2, by vol.).

With cellulose powder (Whatman Chromedia): (n) phenol-water (18:7, w/v); (o) butan-1-ol-acetic acid-water (4:1:5, by vol.); (p) 2-methylbutan-2-ol-5M-formic acid (1:1, v/v); (q) benzene-acetic acid-water (125:72:3, by vol.); (r) sodium formate (5%, w/v)-formic acid (200:1, v/v); (s) ethanol-aq. NH_3 (sp.gr. 0.88)-water (16:1:3, by vol.).

The following compounds were detected by the spray reagents: acids by 0.1% Bromocresol Green in ethanol (Lugg & Overall, 1948); phenolic compounds by diazotized *p*-nitroaniline or sulphanilic acid followed by 10% (w/v) Na_2CO_3 (Smith, 1960); α -carboxypyridines by 3% (w/v) FeSO_4 ; phosphates by a method based on one described by Feigl (1966). The plates were sprayed with ammonium molybdate [5% (w/v) in aq. 35% (v/v) HNO_3], dried at 70°, sprayed again with benzidine-HCl [0.05% in aq. 10% (v/v) acetic acid], dried at room temperature, and sprayed with saturated aq. sodium acetate. Phosphate-containing spots were deep blue.

Radioactive areas on chromatograms were located by radioautography. The t.l.c. plate was carefully placed directly on to the X-ray film (Ilflex No-Screen X-ray film; Ilford Ltd.) in a light-proof holder. The chromatogram was marked in two or three places with radioactive ink to facilitate the location of the radioactive areas on the chromatogram after the film had been developed (Phenisol X-ray developer; Ilford Ltd.).

Experimental procedures

Photoassimilation experiments. Experiments designed to follow the disappearance of aromatic substrates in buffered cell suspensions anaerobically in the light were normally performed in 100 ml. conical flasks equipped with gas inlet and outlet tubes, the latter being led into a 20 cm. head of water to provide a slight positive pressure within the system. The vessels were shaken in a Warburg apparatus fitted with a Perspex water bath at 30°, illuminated from beneath by a bank of 40 w tungsten lamps (B. Braun, Melsungen, W. Germany).

Short-exposure experiments with radioactive benzoate. The apparatus for short-exposure experiments was similar to that described by Knight (1962). The vessel was constructed from a 100 ml. medical flat bottle, the neck of which was attached by a thick rubber tube to an assembly of inlet and outlet tubes for a cooling system and gas. The outflowing gas was led through a trap containing M-NaOH (50 ml.) and into a cylinder of water about 30 cm. in depth. A hole was cut into the bottom of the bottle to accommodate a tap (4 mm. bore) for taking samples, and a second hole, through which additions were made, was cut into the shoulder. The flask was illuminated from the front by a 500 w Photoflood

lamp (Phillips Electrical Ltd., London) placed 20 cm. from the vessel. The contents of the vessel were stirred vigorously by a magnetic stirrer revolving on the back wall, and the temperature was maintained at 34° by regulation of the flow rate of tap water through the cooling system. Aluminium reflectors were constructed round the vessel, and these also acted as shields to prevent light shining on the dial of a top-loading balance placed directly under the sampling tap to gauge the approximate weight of the sample taken during the experiment.

Cells grown photosynthetically on benzoate, suspended in 100 ml. of 0.05 M-tris-HCl buffer, pH 7.2 (4–6 mg. dry wt./ml.), were equilibrated in the light under a rapid flow of N_2 (O_2 -free) for 10 min. Benzoate (50 μ moles) was added and was allowed to disappear over the next 32–38 min.; this preincubation period enabled the system to metabolize much smaller amounts of benzoate immediately and rapidly with the result that the specific radioactivity of the labelled benzoate was not diminished by dilution with unlabelled benzoate. At 42 min. a further 2.5 μ moles of benzoate were added, followed 2 min. later by 140–150 μC of [^{14}C]benzoate. Five samples (approx. 20 g.) were taken over the next 60 sec., these being run directly into 250 ml. conical flasks containing 90 ml. of ethanol at –20° to arrest metabolic activity. Each sample was weighed, and all comparisons between samples were made on the basis of radioactivity per unit weight of sample.

After storage overnight at –20° the samples were warmed up to 50°; the insoluble matter was sedimented by centrifugation and extracted by shaking with 80% (v/v) ethanol (100 ml.) at room temperature for 60 min., and again with 96% (v/v) ethanol (100 ml.) for a further 60 min. The insoluble material was designated 'cell debris'. The extracts were combined and concentrated under reduced pressure in a rotary evaporator (temp. <35°) to a volume of about 10 ml. With the removal of the ethanol a quantity of pigmented lipids formed a fine precipitate. This was sedimented by centrifugation at 105000 g for 60 min. at 2° and washed by shaking with 20 ml. of water for 60 min. followed by centrifugation again; the pellet was designated 'lipid fraction'. The supernatant and the washings were combined, acidified to pH <3 with 0.5 ml. of 75% formic acid and extracted three times with 35 ml. of ether. The combined ether extracts were shaken three times with 30 ml. of 4% NaHCO_3 ; the ether-soluble neutral compounds (' E_N fraction') remained in the ether phase whereas the ether-soluble acids (' E_A fraction') were extracted into the NaHCO_3 phase. The NaHCO_3 extract was then acidified (pH <3) with 1.5 M- H_2SO_4 and the E_A fraction extracted back into ether (three times with 70 ml. of ether). The aqueous phase from the first ether extraction was further extracted at pH <3 with ether for 24 hr. in a liquid-liquid-extraction apparatus, the ether extract from this being designated the ' E_{24} fraction' and the aqueous phase the ' W fraction'.

The whole of each E_A fraction was prepared for quantitative application to a chromatogram. To avoid loss of volatile compounds during the evaporation of the large volume of ether, which is significant when only trace quantities are present, the free acids were converted into their non-volatile ammonium salts by aerating for 5 min. with N_2 that had been passed through aq. NH_3 (sp.gr. 0.88). The ether was then evaporated under reduced pressure and the residue acidified with 0.2 ml. of 1.5 M- H_2SO_4 ; the flask was washed

with small volumes of ether until free from radioactive material. The washings (<10 ml.) were combined, concentrated to a volume suitable for chromatography on a warm-water bath and all applied to a silica gel G t.l.c. plate, which was developed two-dimensionally by solvent (a) followed by solvent (c). Each E_{24} fraction was treated similarly although the treatment with $N_2 + NH_3$ was omitted, since there were no significant losses of radioactivity during evaporation of the ether. The E_N fractions were not, after preliminary studies, investigated by chromatography. Each W fraction was evaporated under reduced pressure in a rotary evaporator to a volume of 1–2 ml. A portion of this equivalent to about 3 g. of the original cell suspension was resolved into three main areas by t.l.c. on cellulose powder developed unidirectionally by solvent (n). The frontal area (R_F 0.65–1.0) that ran above the 'tris area' was eluted and separated further by two-dimensional t.l.c. on cellulose powder developed by solvents (p) and (o). The area from the origin to the 'tris area' was designated 'phosphate area'.

Isotope-dilution experiments. Experiments were performed in the same equipment used for the photoassimilation experiments described above. Cells grown photosynthetically on benzoate were suspended in 15 ml. of 0.05 M-tris-HCl buffer, pH 7.2 (8–9 mg. dry wt./ml.). After 10 min. equilibration, with illumination under an atmosphere of argon, [^{14}C]benzoate (5 μ C, 22 μ moles) was added to each flask followed by 0.5 mg. of a possible intermediate in the unlabelled form. After a further 90 min. each cell suspension was centrifuged, the supernatant acidified to pH < 3 with 1.5 M- H_2SO_4 and extracted three times with 15 ml. of ether. The combined extracts were treated in the same way as the E_A fraction in the previous section, followed by two-dimensional t.l.c. on silica gel G developed in both directions by solvent (e). With the less-stable compounds the chromatographic procedure was omitted; isolation was accomplished by addition of more carrier to the extract followed by crystallization of the compound or a suitable derivative.

RESULTS

Under anaerobic photosynthetic conditions, cultures grew on benzoate, *m*-hydroxybenzoate and *p*-hydroxybenzoate, but not on *o*-hydroxybenzoate, protocatechuate or nicotinate. The growth rate was highest on benzoate, and slightly higher on *m*-hydroxybenzoate than on *p*-hydroxybenzoate. The organism did not grow on benzoate, *o*- and *m*-hydroxybenzoate and catechol under aerobic conditions, but grew rapidly on *p*-hydroxybenzoate and protocatechuate.

Aerobic metabolism of p-hydroxybenzoate

Sequential-induction data. The oxygen consumption by *R. palustris* grown aerobically on *p*-hydroxybenzoate with various substrates is shown in Table 1. *p*-Hydroxybenzoate and protocatechuate were immediately oxidized without an induction period, but none of the other compounds stimulated oxygen uptake above that of the

Table 1. *Oxygen consumption with various substrates by R. palustris grown aerobically on p-hydroxybenzoate*

Each Warburg flask contained, in 3.0 ml. of 0.05 M-phosphate buffer, pH 7.2: cells, approx. 8 mg. dry wt./ml.; substrate (added from side arm), 3 μ moles. The centre well contained 0.2 ml. of 20% (w/v) KOH. Temperature, 30°; atmosphere, air.

Substrate	O ₂ consumed (μ l.)	
	After 10 min.	After 20 min.
None	20	39
Catechol	14	31
Benzoate	15	37
2,4-Dihydroxybenzoate	19	37
<i>cis-cis</i> -Muconate	17	40
<i>p</i> -Hydroxybenzoate	117	221
Protocatechuate	86	155

endogenous uptake. Cells grown aerobically on acetate only oxidized *p*-hydroxybenzoate or protocatechuate after a lag period.

Oxidation of protocatechuate by crude cell-free extracts. Extracts from cells grown aerobically on *p*-hydroxybenzoate, supplemented with Fe^{2+} (1 mM), did not oxidize *p*-hydroxybenzoate, but readily oxidized protocatechuate with the consumption of 1 μ mole of oxygen/ μ mole of substrate. The cell-free extracts, prepared by disrupting suspensions (25–35 mg. dry wt./ml.) in 0.05 M-phosphate buffer, pH 7.2, at 0–4° in a Mullard 60 W ultrasonic generator for 20 min., followed by centrifugation at 25 000 *g* for 20 min. at 2°, were a clear pink from the presence of pigmented particles, probably chromatophore fragments. Removal of the particles by further centrifugation at 105 000 *g* for 60 min. at 2° produced a colourless supernatant of similar activity to the 25 000 *g* supernatant, which showed that the enzyme is soluble.

During the oxidation of protocatechuate the reaction mixture became yellow. This colour persisted after the oxygen uptake had ceased, and faded over the following 20–30 min. without further oxygen uptake. If the extracts were dialysed for 18 hr. at 0–4° against tap water and supplemented with Fe^{2+} , the yellow compound accumulated. The material, yellow at neutral and high pH values and colourless at low pH values, possessed spectral properties identical with those described by Dagley, Evans & Ribbons (1960) for γ -carboxy- α -hydroxy-muconic semialdehyde. This structure was confirmed by its property of cyclizing in the presence of NH_4^+ to form a compound possessing spectral and chromatographic properties and colour reactions that were identical with those of 2,4-lutidinic acid. These findings show that the enzyme responsible for ring-fission was a protocatechuate 4,5-oxygenase.

With undialysed crude cell-free extracts the disappearance of protocatechuate and the ring-fission product was accompanied by the transient accumulation of an acid that produced a blue-green colour in the Rothera (1908) test, suggesting the presence of an α -oxo acid. The accumulation was made more complete by the addition of sodium arsenite (Dagley, Chapman, Gibson & Wood, 1964). The 2,4-dinitrophenylhydrazone of the oxo acid was prepared from a reaction mixture that contained, in 8 ml. of 0.05 M-phosphate buffer, pH 7.2: crude cell-free extract (55 mg. of protein), Fe^{2+} (1 mM), sodium arsenite (5 mM) and 30 μ moles of protocatechuate (added in three 10 μ mole amounts, the second and third additions being made after the disappearance of the yellow ring-fission compound). The product had R_F values similar to those of the 2,4-dinitrophenylhydrazone of authentic pyruvic acid, and mixtures of the two derivatives were not resolved by one-dimensional t.l.c. on silica gel G developed by solvents (c), (l) and (m).

Cell-free extracts, with added Fe^{2+} , prepared from cells grown aerobically on acetate or photosynthetically on *p*-hydroxybenzoate were barely active on protocatechuate.

Photometabolism of benzoate and other aromatic compounds

Photoassimilation of aromatic compounds. The ability of cells grown photosynthetically on benzoate to photoassimilate various aromatic compounds is shown in Fig. 1. The following were utilized in order of decreasing rates: benzoate, *m*-hydroxybenzoate, *o*- and *p*-hydroxybenzoate and protocatechuate. Catechol was not utilized over the 4 hr. experimental period; nor were any of the other dihydroxybenzoates except 2,3-dihydroxybenzoate, the uptake of which ceased after a period of slow assimilation. Treatment of the cell suspensions with chloramphenicol (100 μ g./ml.), an inhibitor of protein synthesis, for 15 min. under argon before the addition of the substrates, affected the rates of assimilation of neither the monohydroxybenzoates nor benzoate, indicating that the enzymes involved were already present in the system.

When grown photosynthetically on *p*-hydroxybenzoate, the cells were able to photoassimilate the monohydroxybenzoates and benzoate at similar rates whereas protocatechuate disappeared more slowly (Fig. 2a). Grown photosynthetically on *m*-hydroxybenzoate, the organism was able to utilize benzoate and to a smaller extent protocatechuate (Fig. 2b).

Cells grown photosynthetically on acetate as carbon source were barely active on benzoate, *m*-hydroxybenzoate and *p*-hydroxybenzoate.

Fig. 1. Photoassimilation of various substrates by *R. palustris* grown photosynthetically on benzoate. Each flask (100 ml.) contained, in 30 ml. of 0.05 M-tris-HCl buffer, pH 7.2; cells, 5–6 mg. dry wt./ml. and substrate, 20 μ moles. Temperature, 30°; atmosphere, argon. ●, Benzoate; ■, *o*-hydroxybenzoate; Δ, *m*-hydroxybenzoate; ○, *p*-hydroxybenzoate; □, 2,3-dihydroxybenzoate; ▲, protocatechuate; ∇, catechol and the other dihydroxybenzoates.

Effects of air and darkness on the photoassimilation of benzoate. Buffered suspensions of cells, grown photosynthetically on benzoate, were allowed to assimilate benzoate photosynthetically for 45 min., at which time air was admitted to two flasks. One flask was left in the light and the other was removed to another water bath at the same temperature but in the dark. After 40 min. both flasks were returned to photosynthetic conditions by flushing with argon and vigorously shaking each vessel for a few seconds. The results (Fig. 3) show that admission of air in the dark or the light caused an immediate cessation of benzoate utilization. Return to photosynthetic conditions allowed the assimilation of benzoate to proceed unimpaired. A third flask was subjected to a period of darkness under anaerobic conditions, during which time no benzoate was metabolized. On return of the flask to photosynthetic conditions recovery was unexpectedly slow. It was also shown that when the organism was maintained anaerobically in the dark in the absence of benzoate for 40 min. added benzoate was photometabolized normally; but, when benzoate was added before the anaerobic-dark period, benzoate photometabolism was initially impaired. At present there is no explanation for this effect.

Short-exposure experiments. Experiments were performed to follow the time-course of the photoassimilation of benzoate. The patterns of uptake of [*ring*-U- ^{14}C]benzoate (ring experiment) and [*carboxy*- ^{14}C]benzoate (carboxy experiment) were

Fig. 2. Photoassimilation of various substrates by *R. palustris* grown photosynthetically on (a) *p*-hydroxybenzoate or (b) *m*-hydroxybenzoate. Details were as given in Fig. 1.

Fig. 3. Effects of air in the light and in the dark and of darkness under anaerobic conditions on the photometabolism of benzoate by *R. palustris*. Each flask (250 ml.) contained, in 60 ml. of 0.05 M-tris-HCl buffer, pH 7.2: cells, 5–6 mg. dry wt./ml.; benzoate, 55 μmoles. Temperature, 30°; atmosphere, argon. In the period between the arrows (45–85 min.) air was admitted to two flasks, one flask (○) being kept in the light and the other (Δ) in the dark; the third flask (□) was maintained in the dark under argon.

compared. In both experiments there was rapid incorporation of ^{14}C into the cell debris and W fraction, with a relatively low influx into the lipid fraction and the E_{24} fraction. Incorporation of ^{14}C into the E_N fraction was also low in both

experiments and the presence of a small amount of lipid, not removed during the centrifugation procedure, probably accounted for the major part of it; the addition of catechol (in the ring experiment) to the ether as a carrier followed by shaking with saturated lead acetate solution, which removed the catechol into the aqueous phase, caused no significant decrease in the radioactive content of the ether phase, indicating the absence of labelled catechol.

Chromatography of the E_A fraction revealed four radioactive areas with R_F values lower than the benzoic acid spot, but which contained only little radioactivity. The E_{24} fraction also comprised two main areas of activity similar to those of the E_A fraction. The series of radioautograms of the two fractions from the ring and carboxy experiments were similar. Co-chromatography of the separated components of the E_A fraction with all the monohydroxybenzoic acids and dihydroxybenzoic acids showed no coincidence of the labelled compounds with those areas developed by diazotized *p*-nitroaniline, which proved the absence of radioactive hydroxybenzoic acids.

Resolution of the W fraction by cellulose t.l.c. developed by solvent (n) produced three main areas (Fig. 4a): area I, containing phosphates coincident with radioactive areas; area II, containing the 'tris'; area III, in which there was heavy and early incorporation of labelled material, most of which ran close to the solvent front. The components of area III were resolved as shown in Fig. 4(b), the series of radioautograms prepared from the ring and carboxy experiments being again strikingly

similar. The major area A was composed of two close-running spots, and in these experiments was assayed as one; it was clear, however, from the radioautograms that the two components were present in approximately equal amounts throughout the experimental period.

Each radioactive area was carefully removed, eluted into water and assayed; the remaining areas were also assayed so that the total radioactivity on each chromatogram could be determined. The radioactivity of each area was expressed as a

percentage of the total radioactivity, phosphate area included, and this relative radioactivity plotted against time, as shown for the two experiments in Fig. 5(a) and 5(b). In both analyses the curves of the assayed spots behaved similarly; area A possessed negative slopes, which shows that the first detectable water-soluble compound to be formed from benzoate is present in the area; the curves of C both rose to a peak and then fell away, slopes characteristic of early intermediates. The low relative radioactivities of the B and D areas obscured any effect of this kind.

Under the experimental conditions with low concentrations of substrate of high specific radioactivity, the intermediary pool sizes of the water-soluble compounds were generally much larger than those of compounds that were soluble in ether (Table 2).

Isotope-dilution experiments. The identification of labelled compounds possibly involved in the early reactions of benzoate photometabolism was achieved by examining the supernatants of buffered cell suspensions that had been photometabolizing high concentrations of [^{14}C]benzoate for 90 min. in the presence of suspected intermediates (0.5 mg.) acting as carrier. Each carrier compound was tested for incorporation of ^{14}C from [*carboxy*- ^{14}C]benzoate, except with 2-oxocyclohexanecarboxylic acid when [*ring*-U- ^{14}C]benzoate was used.

Cyclohexanecarboxylic acid was separated (Fig. 6) by chromatography and was shown to contain ^{14}C . Further two-dimensional t.l.c. on silica gel G

Fig. 4. Radioautograms of the separation by cellulose t.l.c. of labelled water-soluble compounds from *R. palustris* exposed to [*ring*-U- ^{14}C]benzoate for 14 sec. with illumination under nitrogen. (a) Separation of W fraction into areas I, II and III; solvent (n). (b) Further separation of area III; solvents (p) and (o).

Fig. 5. Time-course of incorporation of (a) [*ring*-U- ^{14}C]benzoate and (b) [*carboxy*- ^{14}C]benzoate by illuminated suspensions of *R. palustris* into W fraction. At zero time 140–150 μC of [^{14}C]benzoate was added to 100 ml. of 0.05 M-tris-HCl buffer, containing cells, 5–6 mg. dry wt./ml., and unlabelled benzoate, 2.5 μmoles . Temperature, 34°; atmosphere, nitrogen. Components: ○, A; ▲, B; □, C; △, D; ●, 'phosphate area'.

Table 2. *Time-course of incorporation of [ring- ^{14}C]benzoate by illuminated suspensions of R. palustris into major components of ether-soluble (E_A plus E_{24}) and water-soluble (W) fractions*

Experimental details are given in Fig. 5.

Time (sec.)	$10^{-3} \times$ Incorporation of ^{14}C (c.p.m./g. of cell suspension)	
	Major components of $E_A + E_{24}$ fractions	Compounds A, B, C and D of W fraction
6	11.4	56.2
14.5	11.4	72.7
23	9.8	76.6
36	7.8	69.1
52	6.1	101.0

Fig. 6. Separation by silica-gel t.l.c. of the compounds labelled in the isotope-dilution experiments. Details are given in Table 4. The components were: E, *trans*-2-hydroxycyclohexanecarboxylic acid; F, unknown; G, pimelic acid; H, benzoic acid; I, 'reduced benzoic acids'.

developed by solvent (d) followed by solvent (k) and by solvent (h) followed by solvent (i) failed to resolve the radioactivity from the acid; the R_F values of cyclohex-1-ene-1-carboxylic acid on these systems, however, are similar to those of cyclohexanecarboxylic acid. The radioactive material was diluted with 532mg. of cyclohexanecarboxylic acid, which was converted into the amide (m.p. 186–187°; reported m.p. 185–186°) and crystallized from water, ethanol, ethanol–water and ethanol–ether to constant specific radioactivity (Table 3). The value was about 25% of that expected if all the labelled material isolated was associated with cyclohexanecarboxylic acid.

Cyclohex-1-ene-1-carboxylic acid was similarly separated (Fig. 6) and the area it occupied on the

chromatogram was shown to be strongly and coincidentally labelled. The material was diluted with 950mg. of cyclohex-1-ene-1-carboxylic acid and from this the amide (m.p. 129–131°; reported m.p. 127–128°) and the *trans*-2-bromocyclohexanecarboxylic acid (m.p. 109°; reported m.p. 108°) were prepared. After repeated crystallization of the amide from water and ethanol, and of the hydrobromide from light petroleum (b.p. 60–80°) mixed with benzene, ethanol or ether, both compounds had the same molar specific radioactivity, the value being about 75% of that expected if all the radioactivity isolated was due to cyclohex-1-ene-carboxylic acid (Table 3).

Cyclohex-2-ene-1-carboxylic acid was, owing to its instability, not separated by chromatography. A 300mg. sample of the acid was added directly to the ether extract, the ether was evaporated and the acid dissolved in a little chloroform. A slight excess of bromine was added to form 2,3-dibromocyclohexanecarboxylic acid (m.p. 167–169°; reported m.p. 167°), which was thoroughly washed with water and crystallized from 90% formic acid to constant specific radioactivity. As shown in Table 3, the specific radioactivity was lower than that of either of the above compounds, the total radioactivity being about 6% of that located in the cyclohexane-1-carboxylic acid.

Cyclohex-3-ene-1-carboxylic acid was treated similarly and was brominated to give 3,4-dibromocyclohexanecarboxylic acid (m.p. 81°; reported m.p. 85°). After four crystallizations the compound contained no detectable radioactivity.

trans - 2 - Hydroxycyclohexanecarboxylic acid, separated by chromatography (Fig. 6) from the other ether-soluble material, was coincident with a radioactive area, and further two-dimensional t.l.c. on silica gel G developed by solvent (c) followed by solvent (l), and by solvent (j) followed by solvent (b), failed to resolve the carrier and radioactivity. *trans* - 2 - Hydroxycyclohexanecarboxylic acid (200mg.) was added and this was crystallized to constant specific radioactivity from ether and benzene, and afterwards its *p*-bromophenacyl ester (m.p. 139–140°) was crystallized from ethanol, ethanol–water, ethanol–benzene and methanol; both compounds possessed the same molar specific radioactivity, the value of which (Table 3) showed that all the radioactivity isolated with the carrier was *trans*-2-hydroxycyclohexanecarboxylic acid.

2-Oxocyclohexanecarboxylic acid (300mg.) was added directly to the ether extract. The acidic compounds were separated from any labelled neutral compounds such as cyclohexanone by extraction with 4% sodium hydrogen carbonate followed, after acidification, by extraction back into ether. The ether was evaporated and the acid mixture heated at 85° for 20min. to decarboxylate

Table 3. Crystallization of carrier compounds or their derivatives to constant specific radioactivity after reisolation from illuminated suspensions of *R. palustris* grown photosynthetically on benzoate

		Observed specific radioactivity (c.p.m./μmole)									
Carrier compound	Expected specific radioactivity (c.p.m./μmole)	No. of times crystallized									
		1	2	3	4	5	6	7	8	9	
Cyclohex-1-ene-1-carboxylic acid	126	93.7	98.0	93.5	93.2	94.8	—	—	—	—	
		—	—	95.6	—	—	95.9	—	—	—	
Cyclohex-2-ene-1-carboxylic acid	Not known	—	—	—	18.3	18.2	—	—	—	—	
Cyclohex-3-ene-1-carboxylic acid	Not known	—	—	—	0.0	0.0	—	—	—	—	
Cyclohexanecarboxylic acid	150	41.7	39.0	38.1	38.0	37.8	—	—	—	—	
2-Hydroxycyclohexanecarboxylic acid	56.7	58.6	59.1	59.5	—	—	58.3	57.9	—	—	
		—	—	58.4	—	—	—	55.0	—	—	
2-Oxocyclohexanecarboxylic acid	Not known	5.57	4.8	—	5.15	—	—	—	4.2	4.24	
Pimelic acid	98.6	88.5	94.8	95.3	94.7	92.8	94.0	—	—	—	
		—	—	96.4	—	100.3	98.0	—	—	—	

Each carrier (0.5 mg.) was incubated for 90 min. in 0.05 M-tris-HCl buffer, pH 7.2, containing: cells, 8–9 mg. dry wt./ml., and [carboxy-¹⁴C]benzoate, 5 μC (22 μmoles). With 2-oxocyclohexanecarboxylic acid [ring-U-¹⁴C]benzoate was used at the same concentration. Temperature, 30°; atmosphere, argon.

the 2-oxocyclohexanecarboxylic acid to form cyclohexanone, from which cyclohexanone thiosemicarbazide was prepared (m.p. 159–160°; reported m.p. 165–167°); the thiosemicarbazide of authentic cyclohexanone and a mixture of both preparations also had m.p. 159–160°. The crude product was thoroughly washed with water and then crystallized to constant specific radioactivity. As shown in Table 3, the value was low but the radioactive compound co-crystallized with the cyclohexanone thiosemicarbazide from water, ethanol, ethanol–water and benzene.

Pimelic acid was separated by chromatography (Fig. 6) and was found to be coincidentally labelled. Further two-dimensional t.l.c. on silica gel G developed by solvent (c) followed by solvent (k), and by solvent (j) followed by solvent (f), and on cellulose powder developed by solvent (o) followed by solvent (s), and by solvent (q) followed by solvent (t), did not separate radioactivity from the pimelic acid. Further pimelic acid (200 mg.) was added to the radioactive material and was crystallized from water, benzene and benzene–ethanol, and afterwards its di-*p*-bromophenacyl ester (m.p. 137–138°; reported m.p. 136–137°) was prepared and crystallized from ethanol, benzene, ethanol–benzene and chloroform; both compounds possessed the same molar specific radioactivity, the value of which showed that virtually all the radioactivity isolated was associated with pimelic acid (Table 3).

Neither 2-oxopimelic acid (isolated from the reaction mixture as the 2,4-dinitrophenylhydrazine) nor *trans*-4-hydroxycyclohexanecarboxylic acid, as shown by radioautography, was detectably labelled.

Attempted preparation of cell-free extracts active on benzoate. Suspensions (40–50 mg. dry wt./ml.) of cells grown photosynthetically on benzoate in 10 ml. of 0.05 M-tris or 0.05 M-phosphate buffer, pH 7.2, were disrupted at 0–4° in a Mullard 60 W ultrasonic generator for 10 min. or in a Hughes (1951) press. The disrupted cell suspensions were centrifuged at 25 000 *g* for 15 min. at 2° to remove the cell debris, producing a clear deep-red supernatant. Addition of the supernatant to a vessel illuminated under nitrogen at 30° and containing 20 μ moles of benzoate in 20 ml. of 0.05 M-tris or 0.05 M-phosphate buffer, pH 7.2, failed to cause any detectable breakdown of benzoate. The addition of various combinations of Fe²⁺, Mg²⁺, NADH, ATP and GSH made no difference. Extracts that had been prepared under nitrogen were also inactive, even when supplemented with these cofactors, as were the preparations that were added directly after the ultrasonic treatment or still frozen from the Hughes press, with the centrifugation procedure omitted. Extracts from which the chromatophore fragments had been removed by centrifugation

under nitrogen at 105 000 *g* for 90 min. at 2° and supplemented with Fe²⁺, Mg²⁺ and ATP did not dissimilate benzoate.

It was subsequently found that the addition of small amounts of disrupted cell suspensions to whole cells actively photometabolizing benzoate caused a drastic decrease in the rate of benzoate utilization (Dutton & Evans, 1968a).

DISCUSSION

Aerobic metabolism of p-hydroxybenzoate. The only aromatic compounds found capable of supporting aerobic growth of *R. palustris* were *p*-hydroxybenzoate and protocatechuate; similar observations with strains of the same species have been described by Leadbetter & Hawk (1965) and Hegeman (1967). The intermediates shown to be involved in the aerobic metabolism of *p*-hydroxybenzoate are consistent with the conversion of *p*-hydroxybenzoate into protocatechuate, its cleavage by a protocatechuate 4,5-oxygenase (Dagley *et al.* 1960) to form γ -carboxy- α -hydroxymuconic semialdehyde, and with subsequent breakdown involving pyruvate. The presence of *p*-hydroxybenzoate (or presumably protocatechuate) and oxygen was obligatory for the induction of protocatechuate 4,5-oxygenase.

Hegeman (1967) has reported that extracts from a strain of *R. palustris* grown aerobically on *p*-hydroxybenzoate were capable of oxidizing γ -carboxy- α -hydroxymuconic semialdehyde in an NADP-dependent step to the corresponding muconic acid. This reaction is analogous to the one described by Nishizuka, Ichiyama, Nakamura & Hayaishi (1962) in which extracts from a *Pseudomonas* sp. grown on catechol oxidized α -hydroxymuconic semialdehyde to α -hydroxymuconic acid in an NAD-dependent step; the reaction, however, contrasts with the pathway described by Dagley *et al.* (1964), who showed that extracts from another *Pseudomonas* sp. converted γ -carboxy- α -hydroxymuconic semialdehyde into γ -hydroxy- γ -methyl- α -oxoglutarate and formate in the absence of either NAD or NADP.

Photometabolism of benzoate and other aromatic acids. In earlier studies of the photometabolism of benzoate by a *Rhodospseudomonas* sp., Proctor & Scher (1960) reported that this organism, grown photosynthetically on benzoate, was capable, in the dark, of the aerobic oxidation of benzoate via protocatechuate, catechol and an α -oxo acid. The inference was made (Proctor & Scher, 1960) that molecular oxygen could replace a proposed photosynthetically generated oxidant, assumed to be involved in the photometabolism of benzoate. Evidence that is in conflict with these proposals may be summarized as follows.

(a) The oxygen uptake exhibited by *R. palustris* grown photosynthetically on benzoate, *m*-hydroxybenzoate or *p*-hydroxybenzoate when incubated with various compounds did not reflect the ability of the organism to assimilate the same compounds under photosynthetic conditions. Irrespective of the compound utilized for growth, an oxygen uptake was observed only with *p*-hydroxybenzoate and protocatechuate, significantly the only compounds able to support aerobic growth of this organism (Dutton & Evans, 1967). (b) Protocatechuate 4,5-oxygenase, induced during aerobic growth on *p*-hydroxybenzoate, was virtually absent when the organism was grown photosynthetically on this substrate. (c) The photometabolism of benzoate was totally inhibited by oxygen. (d) Under photosynthetic conditions, cells grown photosynthetically on benzoate did not utilize catechol and utilized protocatechuate only slowly. Protocatechuate also failed to support photosynthetic growth of the organism. (e) The short-exposure experiments with [*carboxy*- ^{14}C]benzoate and [*ring*- $\text{U-}^{14}\text{C}$]benzoate indicated that early decarboxylation does not occur. Patterns of incorporation of ^{14}C from the ring and carboxyl group of benzoate were very similar; further, release of the carboxyl group as carbon dioxide would probably have resulted in a higher incorporation of radioactivity into the 'phosphate area' in the experiment with [*carboxy*- ^{14}C]benzoate (see Knight, 1962). Metabolites such as catechol or any of the monohydroxy- or dihydroxy-benzoates were not detected in these experiments.

Isotope-dilution experiments have led to the identification of several compounds from which the pathway shown in Scheme 1 can be proposed for the photometabolism of benzoate by *R. palustris*. This constitutes a completely new method of aromatic ring metabolism: the aromatic nucleus is first reduced, producing an aliphatic cyclic acid, and it appears that ring-fission and subsequent metabolism is accomplished by a sequence similar to the β -oxidation of fatty acids. Benzoate is reduced to cyclohex-1-ene-1-carboxylate (I). Hydration produces 2-hydroxycyclohexanecarboxylate (II), which is dehydrogenated to form 2-oxocyclohexanecarboxylate (III), and hydration of this results in ring-cleavage and the formation of pimelate (IV). Strong evidence has been presented for the identification of compounds (I), (II) and (IV), whereas radioactive 2-oxocyclohexanecarboxylate was present in such small quantities that the specific radioactivity of the carrier was rather low; however, after its conversion into cyclohexanone, nine crystallizations of the thiosemicarbazide failed to remove the radioactivity. Labelled cyclohex-2-ene-1-carboxylate and cyclohexanecarboxylate were also detected, but in smaller quantities than the

Scheme 1. Proposed pathway for the photometabolism of benzoic acid by *R. palustris*.

cyclohex-1-ene-1-carboxylate (6% and 22% respectively). Neither the nature of the first intermediate nor the roles of cyclohex-2-ene-1-carboxylate and cyclohexanecarboxylate can be decided with any certainty from these experiments; however, it seems reasonable to suppose that the first intermediate is a cyclohexadienecarboxylate, possibly cyclohexa-1,5-diene-1-carboxylate, since cyclohex-3-ene-1-carboxylate was not detectably labelled and cyclohex-2-ene-1-carboxylate may arise as a side product by reduction of the diene in the α -position. Similarly cyclohexanecarboxylate may be a side product, although it is also possible that this compound is a precursor of cyclohex-1-ene-1-carboxylate, benzoate being completely reduced followed by specific dehydrogenation in the α -position. Future work concerned with the identification of the water-soluble compounds, especially the components of area A (Fig. 5b; short-exposure experiments), and the unknown ether-soluble acid (Fig. 6; isotope-dilution experiments), and further studies with cell-free extracts, should lead to a clarification of the early reductive reactions and also those subsequent to pimelate formation. So far, attempts to prepare cell-free extracts capable of benzoate breakdown have failed. This may be connected with the observation (Dutton & Evans, 1968a) that long-chain fatty acid components associated with the pigmented particles in the cell extract, but not completely removed from the extract with the particles, inhibited the photo-assimilation of benzoate by whole cells. The inhibition appeared to be specific to aromatic photometabolism, there being no effect on either photosynthetic growth on acetate or malate, or the aerobic utilization of *p*-hydroxybenzoate. It is known that long-chain fatty acids inhibit a variety of metabolic reactions, including electron transport, but, since saturated fatty acid homologues as low as butyrate and propionate (but not acetate) are

effective inhibitors (P. L. Dutton & W. C. Evans, unpublished work), the cause may be due to competition for cofactors, such as CoA, required by both fatty acid and benzoate metabolism.

In the aromatization of cyclohexanecarboxylate to benzoate by liver enzymes, Babior & Bloch (1966) showed that this reversal of benzoate reduction also involves cyclohex-1-ene-1-carboxylate, but cyclohex-2-ene-1-carboxylate and cyclohex-3-ene-1-carboxylate did not appear to participate directly in the aromatization reactions. It was also shown that the intermediates are present in the form of their CoA thiol esters.

Since the anaerobic breakdown of benzoate appears to be closely associated with the reaction in the light, the reduction of aromatic substrates by *R. palustris* may be catalysed by reductases coupled to a low-redox-potential component of the light-induced electron-transport system, such as ferredoxin. The enzymes involved in the photo-metabolism of aromatic acids are inducible and appear to lack substrate-specificity, as shown by the ability of the organism grown photosynthetically on benzoate or *m*- or *p*-hydroxybenzoate to photo-assimilate a variety of related compounds. Cells grown on *p*-hydroxybenzoate, for example, were able to utilize all the monohydroxybenzoates and benzoate at similar rates.

This novel reductive pathway of aromatic ring dissimilation, employed by *R. palustris* anaerobically in the light, contrasts with the well-known oxidative mechanisms of the obligate aerobic micro-organisms. It seems reasonable to consider that fermentation of aromatic compounds by non-photosynthetic anaerobic micro-organisms, especially the methanogenic bacteria (Clark & Fina, 1952; Roberts, 1962), might also be accomplished by a reductive method of aromatic metabolism.

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