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The Utilization of some Halogenated Aromatic Acids by *Nocardia*

OXIDATION AND METABOLISM

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1. Halogen analogues of *p*-nitrobenzoate and benzoate were oxidized by washed cells of *Nocardia erythropolis*. 2. The oxidation of 2-fluoro-4-nitrobenzoate ceased at the level of acetate, and fluoroacetate was found in the incubation medium and particularly in hot-ethanolic extracts of the cells. 3. Several fluorine-containing intermediates were detected and 2-fluoroprotocatechuate was identified as one of them. 4. The nitro group was also reduced by the organism, as evidenced by the formation of 4-amino-2-fluorobenzoate. 5. Extracts of *N. erythropolis* activated fluoroacetate and condensed the resulting fluoroacetyl-CoA with oxaloacetate to form fluorocitrate. This product was a very powerful inhibitor of citrate metabolism by guinea-pig kidney homogenates and of the aconitase also present in the bacterial extracts. The inhibitions effected by synthetic fluorocitrate and the natural product were comparable. 6. 2-Fluoro-4-nitrobenzoate had negligible mammalian toxicity. 7. The isolation of fluoroacetate as a product of 2-fluoro-4-nitrobenzoate oxidation implies that the aromatic ring in this bacterium must be degraded via a γ-carboxymuconolactone; fluoroacetate cannot arise by metabolism through the isomeric β-carboxymuconolactone.

The observation that washed suspensions of suitably induced cells of *Nocardia erythropolis* oxidized, and accumulated citrate from, 2-fluoro-4-nitrobenzoate, yet failed to grow on this substrate (Smith, Tranter & Cain, 1968), suggested that the organism may convert this fluoro aromatic compound into fluoroacetate. The degradation of other fluorinated carbocyclic and heterocyclic ring compounds by *Pseudomonas* species to this product has been suggested (Behrman & Stanier, 1957; Hughes, 1965), but the evidence for the formation of fluoroacetate is sparse and the mechanisms by which it arises or is further metabolized in these microorganisms has received little detailed attention. This paper describes the oxidation of 2-fluoro-4-nitrobenzoate to fluoroacetate and the enzymic conversion of the latter into fluorocitrate, which is shown to be the ultimate toxic agent. Some comparative experiments with halogenobenzoates are also described.

METHODS AND MATERIALS

Organism, growth methods and preparation of freeze-dried cells and extracts. The organism used, its growth in bulk *p*-nitrobenzoate and benzoate media and the preparation of freeze-dried cells were described in the preceding paper (Smith *et al.* 1968). Extracts were prepared from packed washed-cell suspensions either with the Hughes (1951) press or by ultrasonic disintegration at 20kcyc./sec. for 10min. with the MSE–Mullard 60w instrument. The soluble protein content of these extracts was usually about 15–25mg./ml.

Manometry. Oxidations by washed suspensions were carried out in air by the usual Warburg technique (Umbreit, Burris & Stauffer, 1957) at 30°. Flasks contained: phosphate buffer, pH 7·0, 200μmoles; substrate, 2–10μmoles; cell suspensions (about 10mg. dry wt.), 0·5ml.; KOH (20%, w/v), 0·2ml. in centre well (total volume, 3·0ml.). It was observed that the rate of oxidation of the benzoate and nitrobenzoate analogues by induced cells was markedly increased if the cell suspensions, after harvesting, were incubated with benzoate or *p*-nitrobenzoate (2mM) respectively in buffer for 2–3hr. at 30° before use in manometric experiments. After this preincubation, the cells were centrifuged, washed twice with water at 4° and used immediately.

Chromatography. For paper chromatography of organic acids from incubation mixtures, the following solvent systems were used: *A*, ethanol–aq. NH_3 (sp.gr. 0·88)–water (20:1:4, by vol.); *B*, propan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (6:3:1, by vol.); or *C*, butan-1-ol–acetic acid–water (4:1:5, by vol.). These solvents did not, however, separate acetate from monofluoroacetate. Spots of organic acids were detected by spraying with bromothymol blue made just alkaline with dilute NH_3. In these solvents, 2-fluoro-4-nitrobenzoate and 2-fluoroprotocatechuate ran close together; a search for suitable solvents to improve separation of these compounds showed two, namely solvents *D*, benzene–acetic acid–water (125:72:3, by vol.), and *E*, 5% (w/v) sodium formate–formic acid (200:1, v/v), in both of which 2-fluoro-4-nitrobenzoate ran

near the front while 2-fluoroprotocatechuate had much lower R_F values. Solvent *D* was used as a routine for isolating and purifying 2-fluoroprotocatechuate from incubation mixtures. Phenols and phenolic acids were detected by their fluorescence in u.v. light and by spraying with alkaline diazotized sulphanilic acid or diazotized 4-aminophenyl 2-diethylaminoethyl sulphone (I.C.I. 5091) [1% (w/v) in 10% (w/v) Na_2CO_3] or with 0·5% (w/v) $FeCl_3$ in aq. 50% (v/v) ethanol made slightly acid with dil. HCl. 2,4-Dinitrophenylhydrazone derivatives of keto acids were chromatographed in solvents *F*, 2-methylbutan-2-ol–propan-1-ol–aq. NH_3 (sp.gr. 0·88) (13:1:6, by vol.), *G*, propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (20:1:2, by vol.), and *H*, butan-1-ol–0·5 N-NH_3–ethanol (7:1:2, by vol.), and were detected by their quenching of u.v. light or by spraying with 0·1 N-NaOH.

Acetohydroxamic acid and fluoroacetohydroxamic acid were run in solvents *J*, phenol–water (3:1, w/v), and *K*, ethanol–pyridine–water (3:1:1, by vol.) containing NH_3 (3%, w/v, calculated as the gas) (Bergman & Segal, 1956). They were detected by spraying with $FeCl_3$, after which they appeared as reddish-purple spots on a yellow background. Runs were performed in either ascending or descending directions on Whatman no. 1 or no. 4 paper as indicated in the text.

Citrate and fluorocitrate were separated on a short (10 cm. × 1 cm.) column of Amberlite IRA-400 ion-exchange resin (chromatography grade) prepared as described by Peters, Wakelin & Buffa (1953) and used in the Cl^- form. Citrate was eluted by 0·02 N-HCl in fractions 10–16 (5 ml. fractions), but fluorocitrate was not eluted until the HCl concentration was raised to 0·1 N. It appeared in fractions 4–9 after increasing the HCl concentration. Trial separations of known amounts of citrate and synthetic fluorocitrate gave recoveries of the latter in excess of 90%.

Fluoroacetate was detected by gas–liquid chromatography of the free acid in ethanol or ether solutions dried over anhydrous Na_2SO_4. The instrument used was a Panchromatograph model (W. G. Pye and Co. Ltd., Cambridge) with a 5 ft. glass column (4 mm. internal diam.) of acid-washed Celite (60–70 mesh) coated with 9·2% (w/w) polyethylene glycol succinate and 0·8% (w/w) H_3PO_4. The column was injected (1 μl. samples) with a Hamilton (Whittier, Calif., U.S.A.) micro-syringe and its temperature maintained at 135°; the carrier gas was nitrogen at a flow rate of 50 ml./min. A flame ionization detector set to high sensitivity (amplification 1×10^{-9} or 3×10^{-10}) was used to detect the separated components. Peak areas in quantitative experiments were measured by multiplying the peak height by the width at half the height (Cremer & Muller, 1951). With the nearly Gaussian curves of the fluoroacetate peaks, this method gives results corresponding to 0·84 × those found by integration.

Chemicals. Most of the chemicals used were recrystallized commercial samples. 2-Bromo-4-nitrobenzoic acid (m.p. 165°), 2-fluoro-4-nitrobenzoic acid (m.p. 176°) and 2-iodo-4-nitrobenzoic acid (m.p. 140°) were synthesized from the corresponding halogenated nitrotoluenes (available from Koch–Light Laboratories Ltd., Colnbrook, Bucks.) by oxidation with excess of neutral aq. $KMnO_4$ (Schmelkes & Rubin, 1944). In this heterogeneous reaction good yields of the acids were obtained only if the flask contents were mechanically stirred during the oxidation. 3-Fluoro-4-nitrotoluene was prepared from *m*-fluorotoluene (Koch–Light Laboratories Ltd.) by nitration with fuming HNO_3 (sp.gr. 1·51) by the method of Schiemann (1929). The 3-fluoro-4-nitrotoluene, m.p. 50°, was then oxidized to 3-fluoro-4-nitrobenzoic acid, m.p. 175°, by neutral $KMnO_4$ (Schmelkes & Rubin, 1944). 4-Amino-2-fluorobenzoic acid was prepared by alkaline-$FeSO_4$ reduction of the corresponding nitro compound (Cartwright & Cain, 1959*b*). Succinyl-CoA, acetyl-CoA and fluoroacetyl-CoA were synthesized from CoA and the respective anhydrides by the methods of Simon & Shemin (1953), Lipmann & Tuttle (1945) and Marcus & Elliott (1956) respectively. Sodium fluorocitrate (synthetic) was a gift from Sir Rudolph Peters.

Determinations. Arylamines were determined by the diazotization method of Glazko, Wolf & Dill (1949) with *p*-aminobenzoate and 4-amino-2-fluorobenzoate standards. Ammonia was determined by nesslerization after distillation into dilute H_2SO_4 in Conway units (Cartwright & Cain, 1959*a*). Catechol substances were determined by a quantitative modification of the method of Evans (1947). Succinate was determined manometrically, after extraction into ether and subsequent removal of the ether, with a succinoxidase preparation from ox heart muscle (Umbreit *et al.* 1957); β-oxoadipate and acetate were not oxidized by this preparation. Citrate was estimated by the methods of Saffran & Denstedt (1948) and of Taylor (1953). The latter was particularly useful for estimating small amounts (less than 0·5 μmole) of citrate and also for fluorocitrate, which gives very low readings (approx. one-fortieth on a molar basis compared with citrate) by the Saffran & Denstedt (1948) method. Fluoroacetate was estimated in aqueous solutions by the thiosalicylate method of Ramsey & Patterson (1951). Acetyl-CoA and fluoroacetyl-CoA were estimated by the change in E_{232} (ϵ 4500) brought about by arsenolysis in the presence of phosphotransacetylase (Stadtman, 1957), and as their hydroxamate derivatives by the method of Lipmann & Tuttle (1945). Their activity for use in the bacterial system was checked first with purified citrate synthase (Boehringer). The F^- ion was detected by the paper-chromatographic method of Hall (1957) and within the range of 1–8 μg./ml. estimated by the sensitive and highly specific method of Belcher, Leonard & West (1959*a*,*b*), which involves the formation of a blue ternary complex by the fluoride with alizarin complexan (3-aminomethyl-*NN*-dicarboxymethyl-1,2-dihydroxyanthraquinone) in the presence of Ce^{3+} or La^{3+} ions.

Organic fluorine was estimated quantitatively by the spectrophotometric method of Hall (1963), which can readily and reproducibly measure submicrogram amounts of fluorine in biological material. With this sensitivity, the maintenance of strict cleanliness and fluoride-free reagents, as advocated by Hall (1963), was absolutely essential. Organic material was ashed in platinum crucibles, the resulting fluoride collected by diffusion as HF and the complex formed with lanthanum alizarin complexan extracted into a butan-2-ol–hydroxylamine solvent and its E_{570} value measured as described by Hall (1963). Where bands of suspected fluorine-containing compounds on developed chromatograms were tested for fluorine, two control strips of similar size from other parts of the same sheet were always estimated simultaneously. Similarly, the fluorine estimations on 2-fluoroprotocatechuate (see below) were measured against similar determinations on recrystallized protocatechuic acid to provide blank values. Mr R. J. Hall of the National Agricultural Advisory Service,

Ministry of Agriculture, Fisheries and Food, Kenton Bar, Newcastle upon Tyne, kindly carried out most of our quantitative organic fluorine analyses himself.

Enzyme assays. Acetate-activating enzyme [acetate–CoA ligase (AMP), EC 6.2.1.1] was assayed by measuring the rate of formation of acetohydroxamate by the method of Lipmann & Tuttle (1945).

Citrate synthase [citrate oxaloacetate-lyase (CoA-deacylating), EC 4.1.3.7] was assayed by one of three methods: (i) by following the decrease in E_{232} due to the disappearance of acyl-CoA in the presence of oxaloacetate (Dixon & Kornberg, 1959); (ii) by the increase in E_{340} due to the reduction of NAD^+ when excess of malate and malate dehydrogenase were coupled with citrate synthase in the presence of acetyl-CoA or an acetyl-CoA-generating system (CoA, acetyl phosphate and phosphotransacetylase) (Stern, Ochoa & Lynen, 1952); or (iii) by estimating by Taylor's (1953) method the citrate product formed in this reaction when partially purified preparations of citrate synthase were used.

The enzyme system converting β-oxoadipate into succinate and acetyl-CoA was assayed by following the disappearance of substrate manometrically by catalytic decarboxylation with 0·1 M-4-aminoantipyrine (Cartwright & Cain, 1959*a*).

Acyl-CoA hydrolase (EC 3.1.2.1), aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3], fumarase (L-malate hydro-lyase, EC 4.2.1.2), malate dehydrogenase (L-malate–NAD^+ oxidoreductase, EC 1.1.1.37) and the two isocitrate dehydrogenases [L_s-isocitrate–NAD^+ (or $NADP^+$) oxidoreductases (decarboxylating), EC 1.1.1.41 and EC 1.1.1.42] in the bacterial extracts were measured spectrophotometrically by the methods summarized by Polakis & Bartley (1965), but at pH 7·0.

All spectrophotometric measurements were made with a Unicam SP. 800 recording spectrophotometer fitted with the SP. 820 constant-wavelength attachment and a constant-temperature cuvette holder maintained at 30°.

Succinate dehydrogenase [succinate–(acceptor) oxidoreductase, EC 1.3.99.1] was determined by following either the reduction of 2,6-dichlorophenol-indophenol, as shown by the decrease in E_{600} (Ells, 1959), or oxygen uptake in the Warburg apparatus, in the presence of succinate (3·3 mM), KCN (3·3 mM), phenazine methosulphate (0·8 mM) and tris–HCl buffer (0·05 M).

β-Oxoadipate thiophorase (succinyl-CoA–3-oxoadipate CoA-transferase, EC 2.8.3.6) and β-ketothiolase (acyl-CoA–acetyl-CoA *C*-acyltransferase, EC 2.3.1.16) were demonstrated as described by Katagiri & Hayaishi (1957), but with 100 μmoles of β-oxoadipate in a final volume of 1·5 ml.

Enzymes. Phosphotransacetylase (acetyl-CoA–orthophosphate acetyltransferase, EC 2.3.1.8), purified citrate synthase and alcohol dehydrogenase (EC 1.1.1.1) were the products of the Boehringer Corporation, London, W. 5. Malate dehydrogenase (pig heart; 10000 units/ml.) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Metabolism of the halogenonitrobenzoates and halogenobenzoates

The oxidation of the halogenobenzoates was rapidly effected by benzoate-grown washed suspensions of *N. erythropolis*. The ease of oxidation decreased in the order: benzoate > *p*-fluorobenzoate ≫ *o*-fluorobenzoate > *p*-chlorobenzoate > *p*-bromobenzoate > *p*-iodobenzoate; there was frequently a lag before the chloro, bromo and iodo analogues were oxidized if preincubation of the cells with benzoate, just before use, was omitted; the fluoro analogues were always immediately oxidized (Fig. 1).

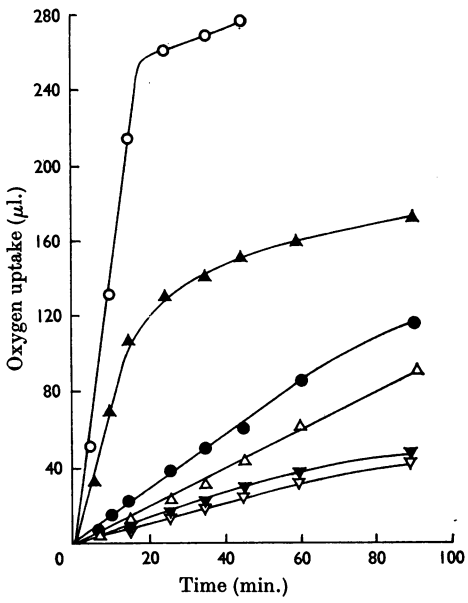


Fig. 1. Oxidation of halogenobenzoates by benzoate-grown *N. erythropolis*. Flasks contained: substrate, 3 μmoles; phosphate buffer, pH 7·0, 200 μmoles; cells, equivalent to 10·2 mg. dry wt.; KOH (20%, w/v), 0·2 ml. in centre well (total volume, 3 ml.). ○, Benzoate; ▲, *p*-fluorobenzoate; ●, *o*-fluorobenzoate; △, *p*-chlorobenzoate; ▼, *p*-bromobenzoate; ▽, *p*-iodobenzoate. Oxygen uptakes were corrected for endogenous respiration (163 μl./hr.).

After growth with *p*-nitrobenzoate, in contrast, washed suspensions of the same actinomycete initially showed quite variable ability to oxidize the 2-fluoro, 2-chloro, 2-bromo and 2-iodo derivatives. The variation was overcome by the preincubation technique described in the Methods and Materials section. After such preincubation, 2-fluoro-4-nitrobenzoate was oxidized (Fig. 2) at about 50% of the rate of *p*-nitrobenzoate itself over the range 0·33–3·3 mM and the 2-chloro derivative at about 25%. The 2-bromo and 2-iodo derivatives were oxidized very slowly (15 and 10% of the rate of *p*-nitrobenzoate in the most active preparations, but frequently less than this), so that it proved difficult to obtain reliable values for total oxygen consumption. Some comparative experiments were

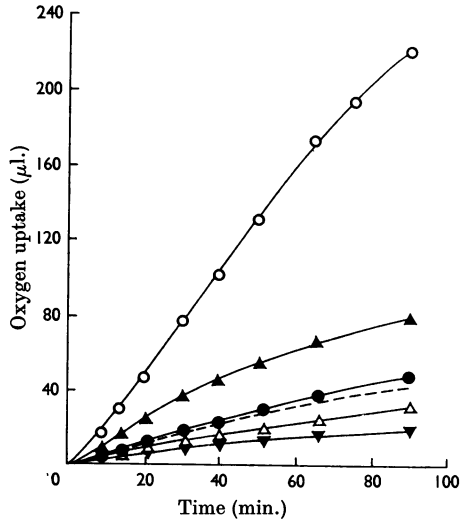


Fig. 2. Oxidation of halogenonitrobenzoates by *p*-nitrobenzoate-grown *N. erythropolis*. Conditions were as given in Fig. 1, but the flasks contained 14·3mg. dry wt. of cells. ○, *p*-Nitrobenzoate; ▲, 2-fluoro-4-nitrobenzoate; ●, 2-chloro-4-nitrobenzoate; △, 2-bromo-4-nitrobenzoate; ▼, 2-iodo-4-nitrobenzoate; ----, 3-fluoro-4-nitrobenzoate. Oxygen uptakes were corrected for endogenous respiration (185μl./hr.)

also performed with 3-substituted analogues; 3-fluoro-4-nitrobenzoate was oxidized much more slowly than the 2-isomer (Fig. 2), but 3-methyl-4-nitrobenzoate was oxidized at about half the rate of *p*-nitrobenzoate.

In several experiments with *p*-nitrobenzoate and its 2-fluoro analogue the flask contents at the end of oxidation were analysed for ammonia, in which form the nitrogen of the nitro group was known to appear (Cartwright & Cain, 1959*a*). In these experiments 2-fluoro-4-nitrobenzoate took up an average of 3·9 moles of oxygen/mole of substrate oxidized; this was about 80% of that found for *p*-nitrobenzoate itself (Cartwright & Cain, 1959*a*), but slightly in excess of that required to oxidize 2-fluoro-4-nitrobenzoate to fluoroacetate in accordance with eqn. (2):

$$p\text{-}O_2N\cdot C_6H_4\cdot CO_2H + 5\tfrac{1}{2}O_2 \rightarrow 7CO_2 + NH_3 + H_2O \quad (1)$$

$$2\text{-}F\text{-}4\text{-}O_2N\cdot C_6H_3\cdot CO_2H + H_2O + 3\tfrac{1}{2}O_2 \rightarrow CH_2F\cdot CO_2H + NH_3 + 5CO_2 \quad (2)$$

The nitrogen of the nitro group in 2-fluoro-4-nitrobenzoate was recovered entirely as ammonia. An average of 1·02 moles of ammonia/mole of substrate oxidized, as required by eqn. (2), was found over six experiments.

Some metabolites of 2-fluoro-4-nitrobenzoate

4-Amino-2-fluorobenzoate. Both washed suspensions and cell-free extracts of *p*-nitrobenzoate-grown *N. erythropolis* can reduce the nitro group of numerous nitro aromatic compounds (Cain, 1958; Cartwright & Cain, 1959*b*). 2-Fluoro-4-nitrobenzoate (10μmoles) was reduced in the presence of an NADH-generating system of ethanol (20μmoles), alcohol dehydrogenase (200μg., 36 units) and NAD^+ (2μmoles) twice as rapidly as *p*-nitrobenzoate itself by extracts. The reduction product (IV) gave a strong positive reaction in the Bratton–Marshall test for arylamines (Glazko *et al.* 1949) and co-chromatographed with authentic 4-amino-2-fluorobenzoate in solvents *A*, *B* and *C*. Eluates from areas corresponding to that of compound (IV) on unsprayed chromatograms gave u.v. spectra at pH 3·0 and 7·0 identical with those of authentic 4-amino-2-fluorobenzoate, and also gave a strong positive reaction for fluoride after ashing or careful evaporation to dryness and boiling with 0·2ml. of 63% (w/v) potassium hydroxide for 1hr. (Aldous, 1963).

Incubation mixtures of 2-fluoro-4-nitrobenzoate in phosphate buffer, pH 7·0, and washed-cell suspensions (Cartwright & Cain, 1959*b*) yielded 4-amino-2-fluorobenzoate in both aerobic and anaerobic (nitrogen atmosphere) incubations. Under aerobic conditions there was approx. 4% conversion of 2-fluoro-4-nitrobenzoate into its reduction product by intact cells compared with 2·5% for *p*-nitrobenzoate itself.

Hydroxy compounds. *p*-Nitrobenzoate is metabolized by *N. erythropolis* through *p*-hydroxybenzoate and protocatechuate to β-oxoadipate (Cartwright & Cain, 1959*a*). Since the same enzymes seemed to be responsible for oxidizing both *p*-nitrobenzoate and its fluoro analogue (Smith *et al.* 1968), it appeared likely that fluoro compounds equivalent to *p*-hydroxybenzoate and protocatechuate might accumulate under conditions where substrate oxidation was restricted by inadequate aeration. The effect of aeration was examined by following the formation of phenolic compounds in incubations performed in 250ml. Erlenmeyer flasks containing about 200mg. of fresh or freeze-dried (*p*-nitrobenzoate-grown) *N. erythropolis*, 50μmoles of 2-fluoro-4-nitrobenzoate and 0·05M-phosphate buffer, pH 7·2 (total volume, 30ml.). One group of flasks was shaken rapidly (360shakes/min.) on a Microid flask shaker, the second slowly (120shakes/min.) in a metabolic shaker and the third group left stationary, all at 30°. The flasks were sampled at intervals, the cell suspension was centrifuged off, and 1ml. portions of the supernatant were tested for *o*-dihydroxyphenolic compounds by the Evans (1947) method. The remaining supernatant, after

acidification, was ether-extracted continuously for 4hr. After evaporation to dryness *in vacuo*, the ether-soluble material was dissolved in a little ethanol and chromatographed in solvents *A* and *C*.

The earlier samples (at 1, 2, 3 and sometimes up to 7hr.) from the stationary cultures (in which the cell suspension settled to the bottom of the flask after about 60min.) frequently showed a spot (I) with a slightly lower R_F value than, but giving the same colour reaction (bright yellow) with diazotized I.C.I. 5091 as, *p*-hydroxybenzoic acid. Eluates of this material from unsprayed portions of similar chromatograms had u.v. spectra at pH7·0 and pH2·0 very similar to those of *p*-hydroxybenzoate. These eluates gave a positive fluorine reaction (Hall, 1957) after alkali treatment (Aldous, 1963), whereas eluates from other parts of the chromatogram were negative. Since no F^- ion was released in the incubation mixtures, this material (I) was believed to be 2-fluoro-4-hydroxybenzoate. Rapidly shaken incubations did not accumulate compound (I).

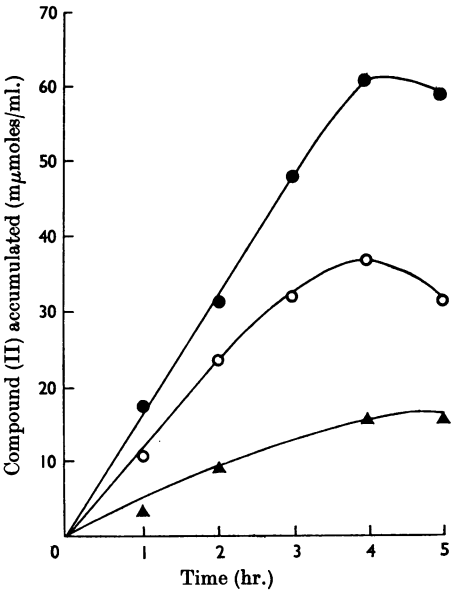
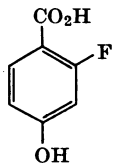


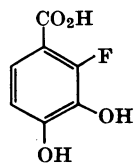
Fig. 3. Accumulation of compound (II) (2-fluoroprotocatechuate) in 2-fluoro-4-nitrobenzoate incubations with washed cells of *p*-nitrobenzoate-grown *N. erythropolis*. Erlenmeyer flasks (250ml.) contained: 2-fluoro-4-nitrobenzoate, 200μmoles; washed cells, approx. 200mg. dry wt.; phosphate buffer, pH7·2, 2·5m-moles (total volume, 50ml.). The flasks were incubated at 30°: ▲, with rapid shaking (360/min.); ○, with slow shaking (120/min.); ●, kept stationary.

The second principal compound (II) was a 'catechol substance' and accumulated best in the stationary incubations of 2-fluoro-4-nitrobenzoate (Fig. 3), where concentrations of 9–10μg./ml. (estimated as protocatechuate) were sometimes recorded. Compound (II) had R_F values 0·24, 0·58, 0·19 and 0·62 in chromatography solvents *A*, *C*, *D* and *E* respectively (authentic protocatechuate has R_F values 0·34, 0·67, 0·25 and 0·56 respectively), but it gave identical colour reactions with ferric chloride (dark green), diazotized I.C.I. 5091 (pale pink) and diazotized sulphanilic acid (deep pink) sprays. Under u.v. light, compound (II) fluoresced strongly with a deep-violet glow, whereas protocatechuate had bright blue fluorescence.

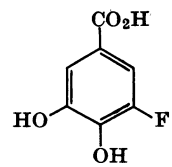
Stationary incubations of *p*-nitrobenzoate-grown cells with 3-fluoro-4-nitrobenzoate and 3-methyl-4-nitrobenzoate also accumulated *o*-dihydroxy compounds (III and V), as evidenced by positive Evans (1947) tests. Chromatography of the derivative (III) from 3-fluoro-4-nitrobenzoate metabolism



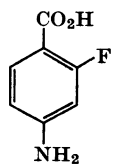
(I) 2-Fluoro-4-hydroxybenzoate



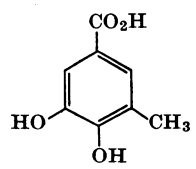
(II) 2-Fluoroprotocatechuate



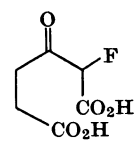
(III) 5-Fluoroprotocatechuate



(IV) 4-Amino-2-fluorobenzoate



(V) 5-Methylprotocatechuate



(VI) 2-Fluoro-3-oxoadipate

gave spots with R_F values 0·08, 0·22 and 0·48 in solvents *A*, *D* and *E*, thus distinguishable from both protocatechuate and compound (II). Eluates of compound (III) also gave a strong positive reaction for fluorine, whereas eluates of (V) derived from 3-methyl-4-nitrobenzoate were negative for fluorine. Compounds (III) and (V) were identical with compound (II) and protocatechuate in their reaction with ferric chloride and diazotized sulphanilic acid.

Isolation and characterization of compound (II). The isolation of compound (II) in quantities sufficient to characterize it was a tedious process because attempts on a larger scale (1–3l. incubations) yielded much lower concentrations. The depth of incubation medium and the 2-fluoro-4-nitrobenzoate concentration (optimum 4mM) could not be varied appreciably from those described for Fig. 3 without a marked decrease in accumulated material. Fresh exponential-phase cells were also essential for good yields in individual incubations.

Accumulation of compound (II) was followed in one of a group of ten identical incubations, and when it reached maximum values all the flask contents were pooled, acidified to pH2·0 with orthophosphoric acid and the cells removed by centrifugation. The acidified supernatant (500ml.) was then concentrated to about 30ml. *in vacuo*, extracted with three 50ml. lots of ether and the ether layers were pooled and evaporated to dryness.

The ether-soluble material was dissolved in ethanol and spread as bands across several wide sheets (30cm. × 35cm.) of Whatman no. 1 chromatography paper. The chromatograms were run in the descending direction in solvent *D*, which gave good separation of compound (II) from the considerable residual 2-fluoro-4-nitrobenzoate and the other fluorescent compounds. Compound (II) was located under u.v. light, the bands were cut out and eluted with ethanol and the ethanol was removed *in vacuo*. The residual material was rechecked for purity by chromatography and crystallized from ethanol–light petroleum (b.p. 68–80°). It had m.p. 178–180°; admixture with an equal amount of authentic protocatechuate (m.p. 194°) gave a large m.p. depression (Found: C, 48·5; H, 3·2; F, 10·6. A fluoro-substituted dihydroxybenzoate, $C_7H_5O_4F$, requires: C, 48·8; H, 2·9; F, 11·0%).

The u.v. spectra of compound (II) showed absorption maxima at: 241 and 284mμ (neutral); 256 and 287mμ (shoulder) (acid); 270 and 294mμ (alkaline). All these maxima are shifted further into the u.v. region compared with the corresponding peaks for protocatechuate.

The i.r. spectrum of compound (II) was compared with those of protocatechuate and 2-fluoro-4-nitrobenzoate and showed a clear absorption band due to C–F at 1040cm.$^{-1}$, absent from protocatechuate but present in the same region in 2-fluoro-4-nitrobenzoate. Other monofluoro-substituted benzoic acids also showed the C–F absorption band within the frequency range 1000–1120cm.$^{-1}$ (Bellamy, 1958; Brügel, 1962). The i.r. spectrum for compound (II) also indicated an aromatic compound (sharp and moderately intense peaks at 1510 and 1610cm.$^{-1}$ due to aromatic C=C stretching vibrations) with phenolic hydroxyl groups (band at 1160cm.$^{-1}$) and a carboxylic acid group (strong absorption peak at 1680cm.$^{-1}$ due to the C=O stretching vibrations in an aryl acid). The ring was disubstituted at the *ortho*-position (band at 772cm.$^{-1}$) and also either 1,2,4-trisubstituted or 1,2,3,4-tetrasubstituted (moderate band at 882cm.$^{-1}$). These bands were also present in the i.r. spectrum of protocatechuate, which differed significantly only in the absence of the C–F absorption band at 1040cm.$^{-1}$.

Proton-nuclear-magnetic-resonance studies on a 15% (w/v) solution of compound (II) in deuterated acetone [99·9% $CD_3 \cdot CO \cdot CD_3$; Ciba (A.R.L.) Ltd., Duxford, Cambs.] were carried out by Mr J. W. Akitt of the Department of Inorganic Chemistry with a Perkin–Elmer R10 spectrometer at 60Mcyc./sec. Peaks in the region 6·5–7·7p.p.m. confirmed the aromatic phenolic nature of compound (II). They exhibited an AB-type spectrum at 7p.p.m. with a $J_{H_1H_2}$ spin coupling constant between the two major peaks of approx. 8·0cyc./sec. Emsley, Feeney & Sutcliffe (1966) give 7·9 ± 0·2cyc./sec. as the theoretical distance in the aromatic nucleus. This demonstration of two unsubstituted ring protons *ortho* to each other confirmed the suspected 1,2,3,4-tetrasubstitution of the nucleus in compound (II). There was also additional complex structure in the spectrum, probably resulting from coupling between the two *ortho*-substituted protons at ring positions 5 and 6 and the fluorine atom at position 2. In particular, a $J_{H,F}$ constant of approx. 2·0cyc./sec. was discernible; Emsley *et al.* (1966) record a value of 2·1–2·3cyc./sec. for the spin–spin coupling constant (J_{HF}) of hydrogen and fluorine *para*-substituted to each other in the aromatic ring. Our spectrum for compound (II) in the region of 7·0p.p.m. showed close similarities to that of 3-fluorocatechol recorded by Goldman, Milne & Pignataro (1967).

Metabolism of 2-fluoroprotocatechuate by cell-free extracts

Extracts of *p*-nitrobenzoate-grown cells of *N. erythropolis* or *p*-hydroxybenzoate-grown *Pseudomonas* C1 (a departmental isolate) rapidly oxidized protocatechuate quantitatively to β-oxoadipate (Cartwright & Cain, 1959*a*) and also attacked 2-fluoroprotocatechuate, though much more slowly (Table 1). 2-Fluoroprotocatechuate

Table 1. *Oxidation of protocatechuate, 2-fluoroprotocatechuate and 5-fluoroprotocatechuate by extracts of* N. erythropolis

Warburg flasks contained: phosphate buffer, pH 7·0, 200 μmoles; substrate, 2 or 5 μmoles; extract, equivalent to 3·3 mg. of protein; KOH (20%, w/v), 0·2 ml. in centre well (total volume, 3 ml.). At the end of oxygen uptake, the incubation was continued for 5 hr. Then KOH was removed carefully, the flask contents were acidified with 0·1 ml. of acetic acid, and 0·4 ml. of 0·1 M-4-aminoantipyrine was added to the side arm. After re-equilibration at 30°, the flask contents were mixed and CO_2 output was measured. Control flasks contained no substrate. All results are corrected for controls (no substrate). 'β-Oxoadipate' is the keto acid formed from 2-fluoroprotocatechuate by catalytic decarboxylation; Cartwright & Cain (1959*a*) established β-oxoadipate as the product of protocatechuate oxidation by *Nocardia* extracts by isolation.

Substrate	Rate of oxidation of substrate (μl. of O_2/hr./mg. of protein)	Oxygen consumed (μmoles)	'β-Oxoadipate' formed (μmoles)
Protocatechuate (2 μmoles)	210	2·0	1·9
Protocatechuate (5 μmoles)	250	4·9	4·8
2-Fluoroprotocatechuate (2 μmoles)	18·5	1·9	1·6
2-Fluoroprotocatechuate (5 μmoles)	23·0	4·7	4·4
5-Fluoroprotocatechuate (2 μmoles*)	5·3	1·6	Not tested

* Calculated from the molar extinction coefficient for protocatechuate.

took up 1 mole of oxygen/mole of substrate oxidized and resulted, on prolonged incubation, in the formation of a keto acid (VI); unlike β-oxoadipate, this product did not give a positive Rothera (1908) reaction (a test that depends on an unsubstituted $-CH_2 \cdot CO-$ grouping), but it did undergo catalytic decarboxylation with 4-aminoantipyrine (Sistrom & Stanier, 1953) and so could be estimated by the same method as β-oxoadipate. The oxidation of 5 μmoles of 2-fluoroprotocatechuate by *Nocardia* extracts yielded 4·7 μmoles of the keto acid after 5 hr. incubation at 30°.

The keto acid, after conversion, with boiling (2 min.), into its 2,4-dinitrophenylhydrazone, chromatographed in solvents *F*, *G* and *H* (descending method) with R_F values 0·46, 0·38 and 0·60 respectively; laevulic 2,4-dinitrophenylhydrazone (derived from β-oxoadipate similarly) had R_F values 0·53, 0·50 and 0·62 in these solvents. The spectrum of the 2,4-dinitrophenylhydrazone of this keto acid in 1·5 N-sodium hydroxide had extinction maxima at 458 and 545–550 mμ (slight shoulder), which distinguished it from the derivatives of pyruvate, α-oxoglutarate and laevulate.

The keto acid produced from 0·5 μmole of 2-fluoroprotocatechuate was converted into its 2,4-dinitrophenylhydrazone and chromatographed as a band on a 30 cm. × 35 cm. sheet in solvent *F*. The dinitrophenylhydrazone band and two blank strips of equal area from the same sheet were cut out, dried in a desiccator and then ashed in platinum crucibles. Fluorine determinations on the residues (Hall, 1963) gave the following results: dinitrophenylhydrazone band, 9·7 μg. of F; blank strip A, 0·4 μg. of F; blank strip B, 0·5 μg. of F. Compound (VI) is therefore probably 2-fluoro-3-oxoadipate, although there was insufficient material for elemental analysis.

When the effect of *Nocardia* extracts (0·8 mg. of protein) on 2-fluoroprotocatechuate (0·3 μmole) was followed spectrophotometrically, a slow reaction (200 min. to completion) was indicated by the appearance of a broad absorption band at 280 mμ; with boiled extracts, no change in the original 2-fluoroprotocatechuate spectrum occurred. It was unlikely that this absorption band was due to a fluorinated β-carboxymuconate because there was no hyperchromic and bathochromic shift on boiling for 30 min., which *cis–cis*-β-carboxymuconate itself invariably exhibited (MacDonald, Stainer & Ingraham, 1954), and which has also been found for *cis–cis*-muconate (Sistrom & Stanier, 1954) and 2-fluoromuconate (Goldman *et al.* 1967).

Extracts of *N. erythropolis*, after it has been grown on *p*-nitrobenzoate or *p*-hydroxybenzoate but not on fumarate, contained the enzyme converting β-oxoadipate into its CoA derivative (specific activity 1·68 μmoles/hr./mg. of protein) and the thiolase cleaving β-oxoadipyl-CoA, in the presence of CoA, to succinyl-CoA and acetyl-CoA (1·32 μmoles/hr./mg. of protein) (Fig. 4). The overall reaction with crude extracts supplemented with succinyl-CoA and CoA yielded succinate and acetyl-CoA, which could be measured as the hydroxamate. Results of a typical experiment are shown in Table 2. The extracts also contain all the enzymes for converting the succinate into oxaloacetate (Table 3).

Conversion of 2-fluoroprotocatechuate into fluoroacetyl-CoA and succinate. If oxidation of 2-fluoroprotocatechuate forms a fluorinated β-oxoadipate, it should be possible to convert this fluorinated keto

acid into fluoroacetyl-CoA (determined as the hydroxamate) and succinate by supplementing the enzymic reaction mixture with catalytic amounts of succinyl-CoA and the other cofactors indicated in Table 2. Three 21 ml.-capacity Warburg flasks with protocatechuate, 2-fluoroprotocatechuate (each at 5 μmoles) and no substrate respectively were each supplemented with CoA, succinyl-CoA, Mg^{2+}, GSH and extract and then incubated. The formation of hydroxamates and succinate are shown in Table 4. The hydroxamate arising from 2-fluoroprotocatechuate was confirmed as fluoroacetohydroxamate by paper chromatography in solvents *J* and *K*. Similarly, that from protocatechuate was identified as acetohydroxamate. The succinate was also confirmed by paper chromatography in solvents *A* and *B*.

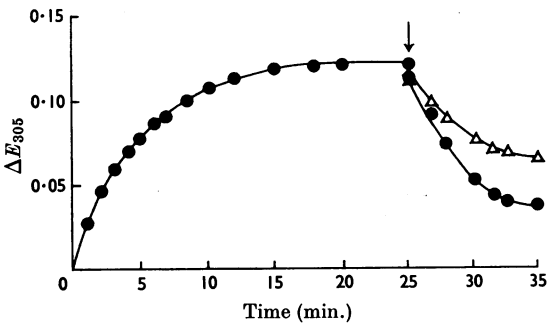


Fig. 4. Spectrophotometric demonstration of β-oxoadipate thiophorase and thiolase in extracts of *N. erythropolis*. Cuvettes contained: potassium β-oxoadipate, 100 μmoles; succinyl-CoA, 0·6 μmole; $MgSO_4$, 1 μmole; tris–HCl buffer, pH 7·5, 200 μmoles; extract, 0·25 mg. of protein (total volume, 1·5 ml.). ●, Formation of β-oxoadipyl-CoA. At the time indicated by the arrow, the reaction was either reversed with 20 μmoles of potassium succinate (●), or the β-oxoadipyl-CoA cleaved to succinyl-CoA and acetyl-CoA by the addition of 0·5 μmole of CoA and 10 μmoles of cysteine (△).

Properties and metabolism of compound (III). Compound (III), the *o*-dihydroxy fluorophenolic acid produced from 3-fluoro-4-nitrobenzoate, had a u.v. spectrum at pH 7·0 very similar to that of protocatechuate. Its u.v. spectrum in alkali showed a broad peak at 290 mμ with only the slightest suggestion of a shoulder at 275 mμ. Both protocatechuate and 2-fluoroprotocatechuate, in contrast, showed two distinct peaks in their spectra within the range 270–310 mμ. Its reaction with ferric chloride, its chromatographic and spectral properties and its origin from a 3-fluoro-substituted aromatic compound make it extremely likely that compound (III) is 5-fluoroprotocatechuate.

Table 2. *β-Oxoadipate metabolism by cell-free extracts of* N. erythropolis

Incubation mixtures contained: tris–HCl buffer, pH 7·6, 150 μmoles; β-oxoadipate, 18·0 μmoles; CoA, 12 μmoles; succinyl-CoA, 0·5 μmole; GSH, 36 μmoles; $MgSO_4$, 10 μmoles; cell extract, 7·2 mg. of protein (total volume, 4 ml.). Incubation was at 30° for 5 hr.

System	β-Oxoadipate disappeared (μmoles)	Acetohydroxamate formed (μmoles)	Succinate formed (μmoles)
Complete	11·2	7·4	4·2
No CoA	0·5	0	0
No succinyl-CoA	0	0	0
Complete, but with boiled extract	0	0	0

Table 3. *Activities of some tricarboxylic acid-cycle enzymes in extracts of p-nitrobenzoate-grown* N. erythropolis

Experimental details are given in the Methods and Materials section. Fluoroacetate at 1·67 mM did not affect the activity of any of these enzymes (compare Table 7).

Enzyme	Substrate	Specific activity (μmoles used/hr./mg. of protein)
Succinate dehydrogenase	Succinate (3·3 mM)	0·43
Succinate dehydrogenase	Succinate (3·3 mM) + malonate (3·3 mM)	0·26
Fumarase	Fumarate (0·33 mM)	16·0
Malate dehydrogenase	Oxaloacetate (0·33 mM)	26·4
Isocitrate dehydrogenase (NADP-linked)	Isocitrate (0·33 mM)	0·51
Isocitrate dehydrogenase (NAD-linked)	Isocitrate (0·33 mM)	0

Table 4. *Formation of acyl-CoA derivatives from their aromatic precursors by extracts of* N. erythropolis

Incubation mixtures in Warburg flasks contained: tris–HCl buffer, pH 7·5, 200 μmoles; substrate (protocatechuate or 2-fluoroprotocatechuate), 5 μmoles; succinyl-CoA, 0·3 μmole; CoA, 2 μmoles; $MgSO_4$, 2 μmoles; GSH, 40 μmoles; extract, 15·2 mg. of protein (final volume, 3 ml.). Control vessels contained no substrate and boiled enzyme. Incubation was at 30° for 6 hr. Oxygen uptake was complete for protocatechuate in 10 min., and for 2-fluoroprotocatechuate in 1·5 hr.

Substrate	Oxygen consumed (μmoles)	Succinate formed (μmoles)	Acylhydroxamate formed (μmoles)
Protocatechuate (5 μmoles)	5·1	1·9	3·6*
2-Fluoroprotocatechuate (5 μmoles)	4·9	1·2	4·7†

* Estimated as acetohydroxamate. † Estimated as fluoroacetohydroxamate.

Samples of compound (III) eluted from chromatograms with ethanol were evaporated to dryness *in vacuo*, and the residual material was taken up in buffer and estimated approximately by the E_{290} value, with protocatechuate (ϵ 3890) as standard. The oxidation of 2 μmoles by fresh extracts of *N. erythropolis* was then followed manometrically. This material was oxidized even more slowly than 2-fluoroprotocatechuate by competent extracts (Table 1), but like its isomer took up approx. 1 mole of oxygen/mole of substrate. The end product was a keto acid that formed a 2,4-dinitrophenylhydrazone with R_F values 0·39 and 0·53 in solvents *G* and *H*.

Fluoroacetate as an end product of 2-fluoro-4-nitrobenzoate oxidation

(*a*) Detection in the incubation medium. The values for total oxygen uptake for 2-fluoro-4-nitrobenzoate oxidation indicated that oxidation ceased at a product at the oxidation level of acetate, whereas previous studies (Cartwright & Cain, 1959*a*) have shown that the non-fluorinated substrate was oxidized to carbon dioxide. Incubation mixtures in which oxidation of these substrates had ceased were thus examined for end products (cf. Behrman & Stanier, 1957).

Washed cells from an exponential-phase culture of the *Nocardia* (grown on *p*-nitrobenzoate) were suspended in 0·05 M-phosphate buffer, pH 7·0, to a final density of 2 mg. dry wt./ml. This suspension was divided into two portions, each 150 ml., in 500 ml. conical flasks; to one was added 100 μmoles of *p*-nitrobenzoate, to the other 100 μmoles of 2-fluoro-4-nitrobenzoate. Both were incubated at 30° with shaking until pilot runs of the same incubations performed in Warburg flasks showed that oxidation was complete. The cells were removed by centrifuging, the neutral supernatants were concentrated to about 10 ml. *in vacuo* and then most of the phosphate was removed by precipitation as calcium phosphate or silver phosphate. The filtrates were passed through a 10 cm. × 1 cm. column of Amberlite IR-120 resin (H^+ form) to remove cations, neutralized with aq. ammonia and evaporated *in vacuo* to about 1 ml. Portions of this solution were then chromatographed in solvents *A* and *B*. No material was detectable from *p*-nitrobenzoate incubations. The 2-fluoro-4-nitrobenzoate incubation yielded only two compounds: a major spot corresponding to fluoroacetate (R_F 0·53 in solvent *A*; R_F 0·62 in solvent *B*) and a much weaker spot running as fluorocitrate (R_F 0·06 in solvent *A*; R_F 0·33 in solvent *B*). Eluates of the material from the larger fluoroacetate spots gave a positive test for fluorine, whereas eluates from blank areas of the same chromatogram were negative.

Similar 2-fluoro-4-nitrobenzoate incubation mixtures at the end of oxidation were acidified with orthophosphoric acid, the cells centrifuged off and the pooled supernatants concentrated to about 50 ml. The concentrated solution was then extracted with 2 vol. of ether and the ether layer re-extracted with 0·1 vol. of 10% (w/v) sodium hydrogen carbonate to recover fluoroacetate. The bicarbonate solution, after neutralization with dilute hydrochloric acid and concentration *in vacuo*, was tested for fluoroacetate by the thiosalicylate method of Ramsey & Patterson (1951) and gave a weak but clearly positive test. Control incubations with either no substrate or *p*-nitrobenzoate and taken through the same procedure were uniformly negative.

Fluoroacetate was confirmed by gas–liquid chromatography of the free acid after extraction into ether from incubation supernatants acidified with orthophosphoric acid. The ether fractions were dried over anhydrous sodium sulphate and carefully evaporated to about 0·2 ml. Samples (1 μl.) were then injected on to the column of a Panchromatograph. The retention time for authentic fluoroacetic acid was 11·2 min. and for the product of 2-fluoro-4-nitrobenzoate metabolism 11·2 min. Material with the same retention time as fluoroacetic acid was found in each of the ether extracts from five separate incubations. On pooling the remaining samples, concentrating and again

chromatographing a 1 μl. sample, a particularly clear peak in the position for fluoroacetic acid was produced. Addition of authentic fluoroacetic acid to a sample of this natural product just before analysis still yielded only a single compact peak on the chart with a retention time of 11·2min. In all except one experiment, there were no other volatile components.

(*b*) Detection in the bacterial cells. If fluoroacetate and acetate arise from their precursors by similar routes (see the Discussion section), they are produced as their CoA derivatives (Katagiri & Hayaishi, 1957) and not as the free acids. It might be expected therefore that the concentration of the free acid in the incubation medium would be low. The cells from the 2-fluoro-4-nitrobenzoate incubations that had shown fluoroacetate in the medium in the previous experiments were harvested by centrifugation, washed once and dropped into hot ethanol. The ethanolic extracts were boiled under reflux at 78° for 15min. to complete hydrolysis of any fluoroacetyl-CoA and the insoluble cell debris was centrifuged off. The ethanol extracts were concentrated *in vacuo* to about 1ml. and reduced to about 0·5ml. with a stream of cold air; they were then accurately adjusted to 0·5ml. with A.R. ethanol. Samples (1 μl.) subjected to gas–liquid chromatography showed very strong peaks for fluoroacetic acid and acetic acid (*A* and *B* in Fig. 5) together with one other major (*C* in Fig. 5) and several smaller unidentified components. No fluoroacetic acid peaks were observed in incubations with *p*-nitrobenzoate and only traces in controls without substrate.

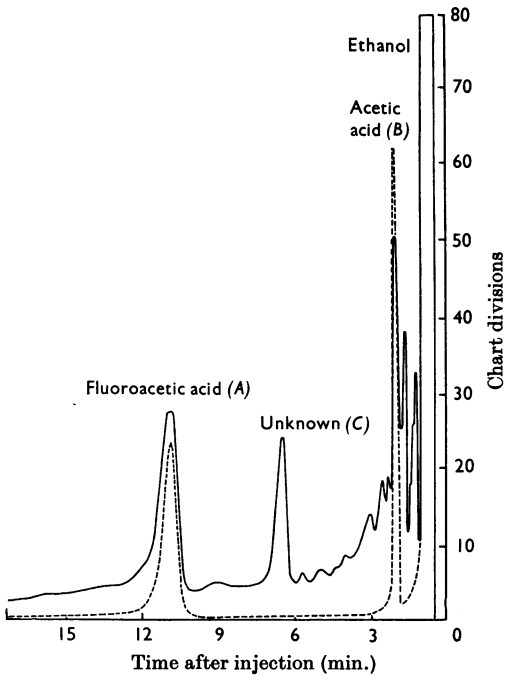


Fig. 5. Chart tracing from gas–liquid chromatography of the hot-ethanol-soluble material from the cells of *N. erythropolis* at the end of oxidation of 2-fluoro-4-nitrobenzoate. The broken line is the tracing of an ethanolic solution of acetic acid and fluoroacetic acid.

A quantitative experiment was performed with the hot-ethanol extracts of identical amounts of cells (200mg. dry wt.) incubated with *p*-nitrobenzoate, its 2- and 3-fluoro isomers, 3-methyl-4-nitrobenzoate (all at 50 μmoles/flask) and a control with no substrate. After concentration and adjusting to 0·5ml. the ethanol extracts (1 μl.) were subjected to gas–liquid chromatography with a detector amplification setting of 1×10^{-9} and the areas of the fluoroacetic acid peaks produced were calculated. The peak areas of fluoroacetic acid produced were: from 2-fluoro-4-nitrobenzoate, 4320mm.^{2}; from 3-fluoro-4-nitrobenzoate, 110mm.^{2}; from the control (no substrate), 70mm.^{2}; from *p*-nitrobenzoate or 3-methyl-4-nitrobenzoate, nil. It is therefore clear that fluoroacetate arose only from 2-fluoro-4-nitrobenzoate. Some experiments were performed with ethanolic extracts of benzoate-grown cells (160mg. dry wt.) incubated with benzoate, *o*-fluorobenzoate, *p*-fluorobenzoate (50 μmoles) or no substrate. Fluoroacetic acid peak areas were nil, 1370mm.^{2}, nil and 25mm.^{2} respectively.

Conversion of fluoroacetate into fluorocitrate

Crude extracts of the organism 'activated' acetate and fluoroacetate as measured by the formation of their hydroxamates (Lipmann & Tuttle, 1945). These hydroxamates were identified as the acetyl and fluoroacetyl derivatives by chromatography with the authentic markers in solvents *J* and *K*. When oxaloacetate was added to such incubations in place of hydroxylamine, material reacting as citrate was formed (Table 5), but fluoroacetate was a poorly accepted substrate for the enzymes in both hydroxamate and 'citrate' formation, and, when present with acetate, markedly decreased citrate synthesis from the latter.

Citrate synthase was present (Fig. 6) in the fraction of crude extracts precipitated by ammonium sulphate between 40% and 70% saturation. Fluoroacetyl-CoA was readily accepted as a substrate for *Nocardia* citrate synthase (Fig. 7) and was metabolized at some 45–50% of the rate of the natural substrate, acetyl-CoA. This was well in excess of the activity suggested by experiments with free fluoroacetate (Table 5), but Marcus &

Table 5. *Acetate and fluoroacetate in 'citrate' synthesis by extracts of* N. erythropolis

Incubations were carried out in tubes shaken at 30° for 2hr. Each tube contained: GSH, 100μmoles; $MgCl_2$, 20μmoles; CoA, 100 Lipmann units; oxaloacetate, 50μmoles; ATP, 25μmoles; tris–HCl buffer, pH 7·3, 600μmoles; cell extract, equivalent to 16·5mg. of protein; other additions, as shown (final volume, 5ml.). Expts. I and II were performed with different enzyme preparations.

	Addition	'Citrate' formed (μmoles)	Excess of citrate over control (μmoles)
Expt. I	None	1·14	—
	Acetate (300μmoles)	2·56	1·42
	Fluoroacetate (300μmoles)	1·25	0·11
	Acetate (300μmoles)+fluorocitrate (2μmoles)*	1·67	0·53
Expt. II	None	0·52	—
	Acetate (300μmoles)	2·11	1·59
	Fluoroacetate (300μmoles)	0·65	0·13
	Acetate (300μmoles)+fluoroacetate (100μmoles)	0·69	0·17

* Corrected for a control containing 2μmoles of fluorocitrate only.

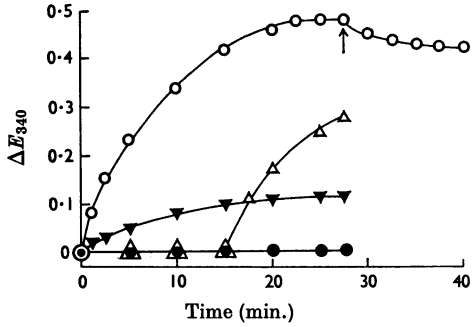


Fig. 6. Citrate synthase in extracts of *N. erythropolis*. Cuvette contents for the complete system were: L-malate, 20μmoles; malate dehydrogenase, 2000 units; NAD^+, 0·5μmole; CoA, 0·1μmole; GSH, 7μmoles; KCl, 25μmoles; KF, 40μmoles; tris–HCl buffer, pH 7·3, 130μmoles; phosphotransacetylase (Boehringer), 30μg. of protein; acetyl phosphate, 1·3μmoles; bacterial extract, 7·65mg. of protein. Reference cuvettes contained all components except NAD^+. ○, Complete system started by the addition of acetyl phosphate at zero time; at 27·5min. (↑) 10μmoles of potassium citrate were added; △, complete system but acetyl phosphate omitted until 15min.; ▼, phosphotransacetylase omitted; ●, malate omitted, or with boiled bacterial extract, or extract omitted.

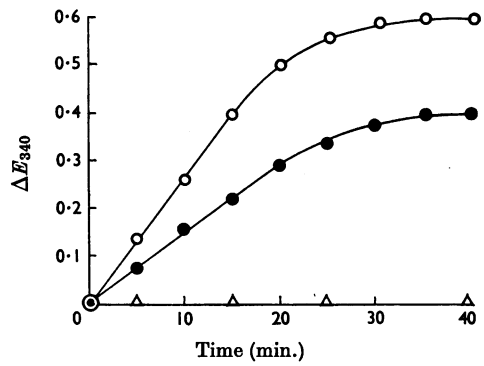


Fig. 7. Utilization of fluoroacetyl-CoA by *Nocardia* citrate synthase. Cuvettes contained: L-malate, 20μmoles; malate dehydrogenase, 2000 units; NAD^+, 0·5μmole; KCl, 25μmoles; K_2SO_4, 40μmoles; KF, 40μmoles; GSH, 7μmoles; tris–HCl buffer, pH 7·3, 150μmoles; acetyl-CoA or fluoroacetyl-CoA, 0·3μmole; bacterial extract, 10mg. of protein. Reference cuvettes contained no NAD^+. ○, System with acetyl-CoA; ●, system with fluoroacetyl-CoA; △, system with either acetyl-CoA or fluoroacetyl-CoA but with boiled extract.

Elliott (1959) have noticed a similar result with mammalian enzymes. Crude extracts also had low but detectable phosphotransacetylase activity because the extracts still slowly synthesized citrate from malate and acetyl phosphate in the absence of added phosphotransacetylase. The presence of weak phosphotransacetylase activity was confirmed by the slow arsenolysis of acetyl-CoA and fluoroacetyl-CoA (Marcus & Elliott, 1959) on addition of bacterial extract.

The product of the *Nocardia* citrate synthase-catalysed condensation of fluoroacetyl-CoA and oxaloacetate reacted as a 'citrate' when estimated by Taylor's (1953) method and co-chromatographed with fluorocitrate in solvents *A* and *C* with R_F values 0·05 and 0·17 respectively. The natural product was readily distinguished from citrate by chromatography on Amberlite IRA-400 resin (see the Methods and Materials section) and it was eluted with 0·1N-hydrochloric acid at the position for fluorocitrate (Fig. 8); this material contained

fluorine. Its identity as fluorocitrate was confirmed by comparing its inhibitory effects on citrate metabolism in guinea-pig kidney cortex with those brought about by authentic fluorocitrate (Table 6) and by examining its effects on the *Nocardia* aconitase (Table 7).

Effect of fluorocitrate and fluoroacetate on aconitase in N. erythropolis

Aconitase activity in crude or ammonium sulphate-fractionated extracts could be demonstrated with either citrate or isocitrate as substrate. In crude extracts, the equilibria at pH 7·2 were strongly in favour of *cis*-aconitate formation from either of these substrates; the hydrolysis of *cis*-aconitate (0·33–10 mM) by *Nocardia* extracts could not be demonstrated spectrophotometrically. The aconitase activity in crude extracts was stimulated 22% by added Fe^{2+} ion (0·01 mM).

Synthetic fluorocitrate very strongly inhibited this enzyme in crude or fractionated extracts and completely suppressed activity with citrate as substrate at 0·1 mM (Table 7); over 300 times as much fluoroacetate was required to bring about comparable inhibition under the same conditions. No enzymic activity was evident with fluorocitrate itself, over the range 1–10 mM.

The natural fluorocitrate, synthesized by *Nocardia* citrate synthase from fluoroacetyl-CoA, also strongly inhibited the *Nocardia* aconitase (Table 7), but did not affect any of the other enzymes listed in Table 3 at a concentration of 0·1 mM.

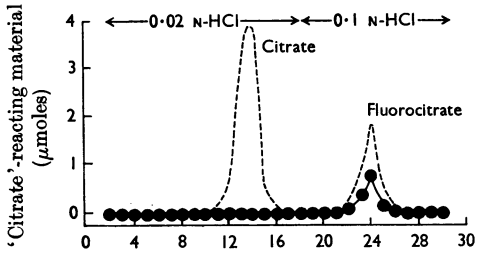


Fig. 8. Chromatography of citrate and fluorocitrate on a 10 cm. column of Amberlite IRA-400 resin. The broken line is the result obtained from the application of a mixture of 9 μmoles of citrate and 4 μmoles of synthetic fluorocitrate; ●, 'citrate'-reacting material derived from the bacterial preincubation mixture (Table 6) containing fluoroacetyl-CoA.

Mammalian toxicity of 2-fluoro-4-nitrobenzoate

Pattison (1959) listed many fluorinated compounds the toxicity of which was related to their ease of metabolism to fluoroacetate (or fluoroacetyl-CoA). The demonstration that 2-fluoro-4-nitrobenzoate was converted into fluoroacetate by micro-organisms led us to check its mammalian toxicity. Serial 1 : 10 dilutions of aqueous sodium 2-fluoro-4-nitrobenzoate (1 M–0·1 mM) were prepared

Table 6. *Inhibition of citrate utilization in guinea-pig kidney homogenates by fluorocitrate and the product of fluoroacetyl-CoA and oxaloacetate condensation by* Nocardia *citrate synthase*

Bacterial preincubation mixture contained: fluoroacetyl-CoA or acetyl-CoA, 1·2 μmoles; L-malate, 80 μmoles; malate dehydrogenase, 8000 units; NAD^+, 20 μmoles; KCl, 100 μmoles; KF, 160 μmoles; K_2SO_4, 160 μmoles; GSH, 30 μmoles; *Nocardia* extracts, 40 mg. of protein (total volume, 4 ml.). Incubations containing fluoroacetyl-CoA, acetyl-CoA and fluoroacetyl-CoA with boiled extract or with no enzyme respectively were incubated at 30°, the reactions being followed spectrophotometrically at 340 mμ to completion. At completion, 0·1 ml. of H_3PO_4 was added to stop the reactions, the precipitated protein was removed and the supernatants were adjusted to pH 7·0 with KOH and then made to 5 ml. with water. Kidney homogenate system contained: potassium phosphate buffer, pH 7·0, containing 0·9% (w/v) KCl, 100 μmoles; $MgCl_2$, 10 μmoles; ATP, 6·0 μmoles; potassium citrate, 10 μmoles; guinea-pig kidney homogenate, equivalent to 200 mg. fresh wt. of kidney. To this system was added 1 ml. of one of the deproteinized bacterial preincubation mixtures. The homogenate system was incubated for 90 min. at 38°, 0·5 ml. of 10% (w/v) trichloroacetic acid was added to precipitate the protein and citrate estimations were made on the supernatants after centrifugation. Expts. (*a*) and (*b*) were performed with different homogenate preparations. Citrate values are corrected for added fluorocitrate where necessary.

Additions to homogenate system	Citrate utilized (μmoles) Expt. (*a*)	Expt. (*b*)
None	8·7	5·2
Bacterial preincubation system containing acetyl-CoA	8·7	—
Bacterial system containing fluoroacetyl-CoA	6·6	2·4
Bacterial system containing fluoroacetyl-CoA but with boiled extract	8·7	5·0
Bacterial system containing fluoroacetyl-CoA but with no extract	8·6	—
Synthetic fluorocitrate (0·2 μmole)	5·6	1·4

Table 7. *Inhibition of aconitase in* N. erythropolis *extracts by fluoroacetate, fluorocitrate and the product synthesized from fluoroacetyl-CoA by bacterial citrate synthase*

Aconitase activity was measured with citrate (1 mM) as substrate in the presence of Fe^{2+} (10 μM). Enzyme and inhibitor were preincubated 5 min. before addition of substrate. Incubation was at 30°. Expts. 1 and 2 were performed with different extract preparations.

	Inhibitor	Concn. of inhibitor (mM)	Aconitase activity (μmoles of *cis*-aconitate formed/hr./mg. of protein)	Inhibition (%)
Expt. 1	None	—	1·86	—
	Fluoroacetate	0·1	1·86	0
		0·33	1·50	19
		1·0	1·08	42
		10·0	0·42	77
	Fluorocitrate (synthetic)	0·010	0·66	65
		0·033	0·30	84
		0·10	0	100
Expt. 2	None	—	1·48	—
	Fluorocitrate (synthetic)	0·017	0·50	67
		0·067	0·12	92
	Fluorocitrate (natural)* from bacterial preincubation mixture:			
	0·1 ml.	0·005†	1·28	13
	0·15 ml.	0·0075	0·61	58
	0·2 ml.	0·01	0·30	80

* The product synthesized from fluoroacetyl-CoA and oxaloacetate by *Nocardia* citrate synthase in Table 6. The material from fluoroacetyl-CoA incubation with the *Nocardia* system with boiled enzyme in Table 6 did not inhibit aconitase.

† Calculated from the amount of NAD^+ reduction in the bacterial incubation mixtures, measured spectrophotometrically.

and 0·5 ml. of the appropriate dilution was injected intraperitoneally into a group of five mice, average weight 27 g. All individuals of the group receiving the 1 M solution died within 5 min., i.e. without the characteristic latent period invariably associated with fluoroacetate poisoning. They also showed none of the cardiac distress or convulsions typical of fluoroacetate toxicity. No individual from any of the other four groups receiving the serial dilutions had died at 21 days. It was concluded that the death of the first group was caused by osmotic effects due to the concentrated solution administered.

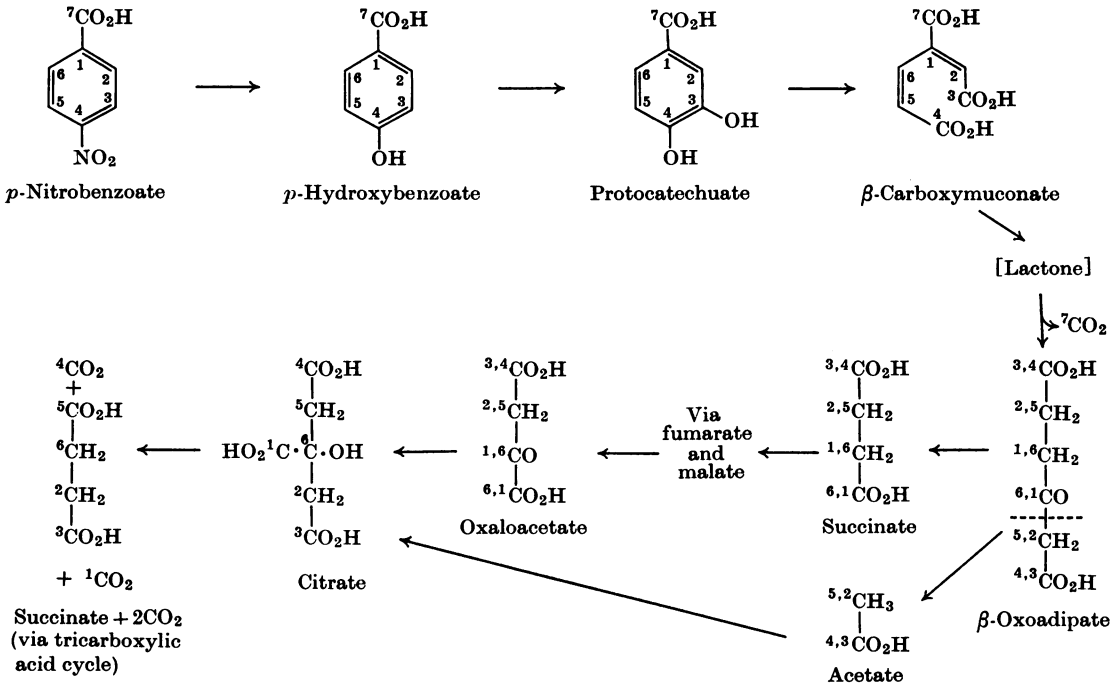
On this basis the LD_{50} of 2-fluoro-4-nitrobenzoate for mice was much greater than 350 mg./kg. body wt., compared with 6·6 mg./kg. for fluoroacetate and 1·35 mg./kg. for 6-fluorohexanoate (Pattison, 1959); i.e. 2-fluoro-4-nitrobenzoate was effectively non-toxic.

DISCUSSION

p-Nitrobenzoate is metabolized by *N. erythropolis* by the route shown in Scheme 1. The fact that the enzymes for its oxidation to carbon dioxide can be induced either by the natural substrate or by halogenated analogues (Smith *et al.* 1968) makes it likely that 2-fluoro-4-nitrobenzoate is degraded via the corresponding fluoro intermediates, at least as far as fluoroacetate.

The original observation that both 2-fluoro-4-nitrobenzoate and fluoroacetate caused citrate accumulation from *p*-nitrobenzoate and the fact that fluoroacetate has now been identified in both the cells and the medium of 2-fluoro-4-nitrobenzoate incubations shows that the C–F bond of the original substrate has remained intact through the many metabolic stages involved in its oxidation to fluoroacetate, and means therefore that the intermediary precursors of fluoroacetate must be fluorinated. This deduction is reinforced by the complete absence of F^- ion released into the incubation medium in any of our experiments, and particularly by the detection of fluorine in the three metabolic intermediates (I), (II) and (III), the probable analogues of *p*-hydroxybenzoate, protocatechuate and β-oxoadipate respectively. Compound (II) has, in fact, been rigorously identified with 2-fluoroprotocatechuate.

Extracts of *Nocardia* have the enzymic complement to degrade β-oxoadipate (as its CoA derivative) to acetyl-CoA and succinate, the stage at which aromatic metabolism is channelled into the terminal oxidation sequences of the tricarboxylic acid cycle. The enzymes for converting succinate into oxaloacetate (succinate dehydrogenase, fumarase and malate dehydrogenase) and for condensing this latter intermediate with acetyl-CoA to form

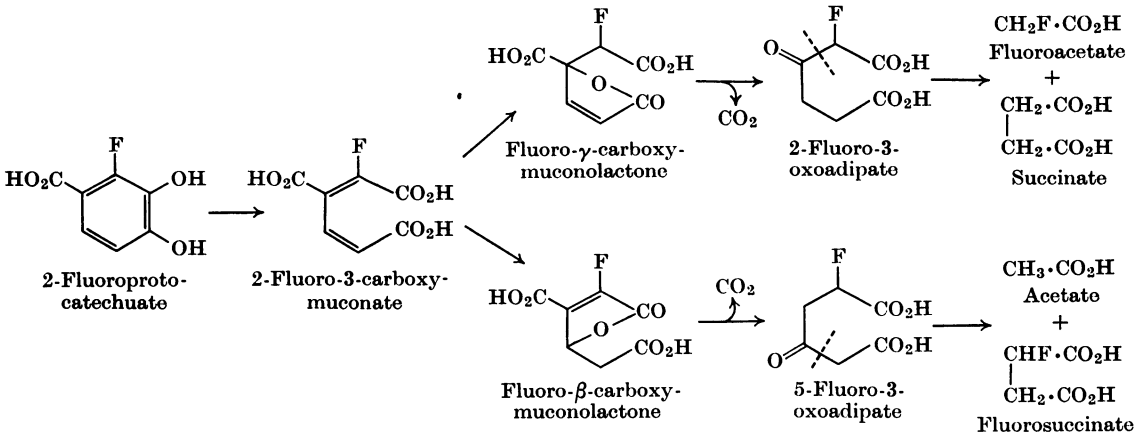


Scheme 1. Fate of carbon atoms in *p*-nitrobenzoate metabolism by *N. erythropolis*.

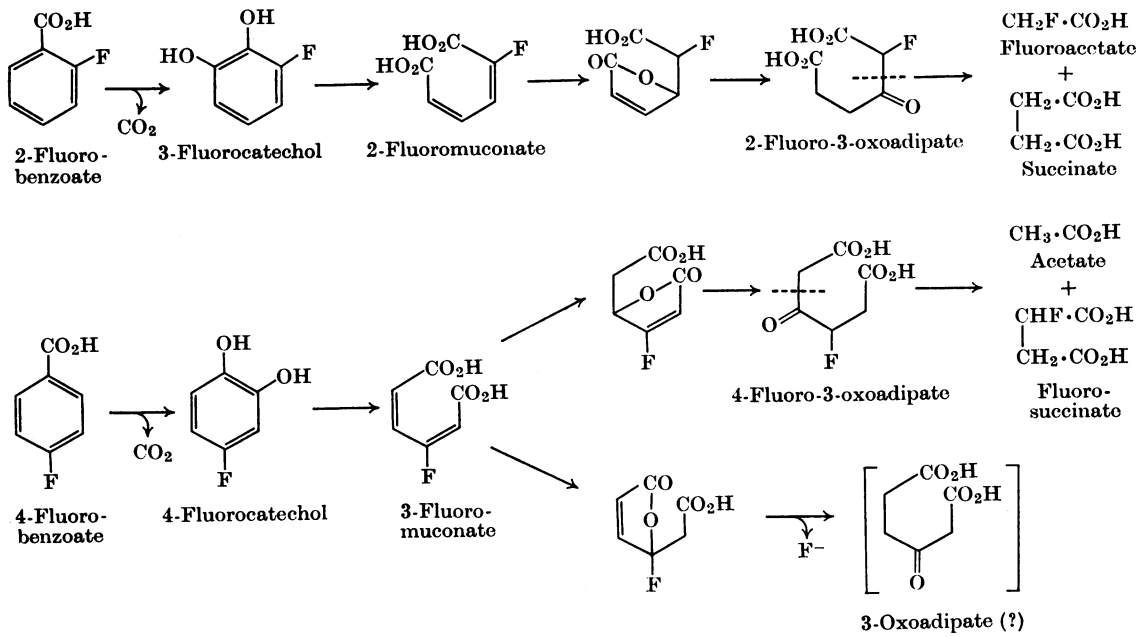
citrate (citrate synthase) are also present, so that *Nocardia* can synthesize citrate from the benzene ring (Scheme 1). Similarly it should be able to form fluorocitrate from the 2-fluoro aromatic substrate. The organism can 'activate' free fluoroacetate (Table 5) and convert this fluoroacetyl-CoA into fluorocitrate (Fig. 7), but fluoroacetyl-CoA is more probably derived directly *in vivo* from the thiolase cleavage of fluorinated β-oxoadipyl-CoA, as evidenced by its enzymic formation from 2-fluoroprotocatechuate, by the large amounts of fluoroacetate found in hot-ethanol extracts of the organism and by the presence of the necessary enzymes in extracts of cells grown with *p*-nitrobenzoate but not succinate. The small yields of fluoroacetate found in the incubation medium probably represent only that portion which has leaked through the cell membrane.

Although metabolism of fluoro analogues and the natural intermediates may parallel each other as far as the (fluoro)citrate stage, oxidation of the fluoro compounds ceases there. The addition of synthetic fluorocitrate to washed or freeze-dried cells oxidizing *p*-nitrobenzoate or citrate leads to rapid and extensive inhibition of oxidation (Smith *et al.* 1968), and we have shown that the natural fluorocitrate synthesized from fluoroacetyl-CoA by extracts of *Nocardia* not only very strongly inhibits the aconitase activity in the same extracts (Table 7) but is itself unable to act as a substrate for aconitase; consequently fluorocitrate is not further metabolized. The lesion in 2-fluoro-4-nitrobenzoate oxidation therefore is at the level of fluoroacetate, from which fluorocitrate is 'lethally synthesized' (Peters, 1952) and inhibits the functioning of the tricarboxylic acid cycle.

Ornston & Stanier (1966) have shown that protocatechuate is converted by *Pseudomonas putida* and *Moraxella calcoacetica* into β-oxoadipate through the unstable γ-carboxymuconolactone. Several other bacterial genera have been found to utilize this γ-isomer (Cain, 1966), whereas all the fungi and yeasts so far examined (Gross, Gafford & Tatum, 1956; Bilton & Cain, 1965) degrade only the corresponding β-carboxymuconolactone. If the fate of C-2 of the aromatic ring is followed in Scheme 1, it is seen to appear in either the acetate or succinate arising from β-oxoadipate. If C-2 is fluorinated, the oxidation of 2-fluoroprotocatechuate should give rise to either 2-fluoro-3-oxoadipate if metabolism is effected via a fluoro-γ-carboxymuconolactone, or 5-fluoro-3-oxoadipate if metabolized through the corresponding β-isomer (Scheme 2). The former keto acid on thiolase cleavage should yield fluoroacetate and succinate; the latter would produce fluorosuccinate and



Scheme 2. Alternative metabolic routes for 2-fluoroprotocatechuate.



Scheme 3. Suggested metabolic routes for 2-fluoro- and 4-fluoro-benzoate (Goldman *et al.* 1967, and this paper).

acetate. The detection of fluoroacetate in ethanolic extracts of the cells and in incubation media and the direct demonstration of fluoroacetohydroxamate and succinate formation from 2-fluoroprotocatechuate by extracts by *N. erythropolis* clearly incriminate the γ-lactone as an intermediate in the degradation of the aromatic ring by this bacterium, and substantiate an earlier suggestion (Cain & Cartwright, 1960) that this lactone is an intermediate in *p*-nitrobenzoate metabolism. These conclusions are reinforced by the observation that no fluoroacetate was formed from 3-fluoro-4-nitrobenzoate. Reference to Schemes 1 and 2 shows that metabolism of this fluoro aromatic compound via the γ-lactone should yield fluorosuccinate instead; 3-fluoro-4-nitrobenzoate can only give rise to fluoroacetate via a β-carboxymuconolactone, which *Nocardia* does not metabolize (Cain &

Cartwright, 1960). Fluorosuccinate is non-toxic (Pattison, 1959), causes no citrate accumulation from *p*-nitrobenzoate and has never been detected in any incubations with *N. erythropolis*.

The metabolism of the fluorobenzoates by *Nocardia* has been examined in less detail because several other workers have largely delineated the general metabolic routes. We were unable to demonstrate even transient accumulation of *o*-dihydric phenols in fluorobenzoate incubations, but Goldman *et al.* (1967) have characterized 3-fluorocatechol and 2-fluoromuconic acid as early intermediates in 2-fluorobenzoate metabolism by a *Pseudomonas*. Our detection of fluoroacetate arising from 2-fluorobenzoate, but not the 4-fluoro isomer, confirms the findings of Goldman *et al.* (1967) because only 2-fluorobenzoate can give rise to fluoroacetate if it is assumed that benzoate and its 2- and 4-fluoro analogues are metabolized by parallel routes (Scheme 3). 4-Fluorobenzoate would form a 3-fluoromuconate, which, after lactonization, would undergo cleavage to fluorosuccinate or alternatively eliminate fluoride at the position of insertion of the carbonyl group. The pseudomonad isolated by Goldman *et al.* (1967) actually eliminated some 80% of organic fluorine during its growth on 2-fluorobenzoate as sole carbon source, a necessary prerequisite if it was not to poison itself with fluoroacetate.

The demonstration that 2-fluoro-4-nitrobenzoate is metabolized to fluoroacetate and leads to citrate accumulation in poisoned incubation mixtures adds another compound to the long list of fluoro derivatives (Pattison, 1959, p. 104 *et seq.*) that are toxic as a result of their metabolism to fluoroacetate. The toxicity of 2-fluoro-4-nitrobenzoate applies only to micro-organisms, however, and indeed only to those genera (predominantly pseudomonads and actinomycetes) that can degrade the (fluorinated) aromatic ring. The inability of the mammalian body to perform this metabolic function (Williams, 1959) except in a few special instances, e.g. 3-hydroxyanthranilate oxygenase (Henderson, 1955; Iaccarino, Boeri & Scardi, 1961), renders 2-fluoro-4-nitrobenzoate non-toxic to mammals, as confirmed by our experiments with mice.

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