approx. 1.28 μ moles or an amount equivalent in F to about 0.2 μ g. of fluoroacetic acid. If we assume a 30% conversion, this would be equivalent to an amount of fluorocitrate of about 0.5 μ g. able to cause this block in the 150 mg. of kidney tissue used.

Analyses of some synthetic fluoro fatty acids by reversed-phase chromatography showed that the presence of fluorine made the fatty acids appear in the fraction with 3 carbon atoms less than the non-fluorinated and saturated fatty acids, i.e. C₁₈ ran with C₁₅, C₁₂ with C₉ (Fig. 2). At this stage, it appeared therefore that the fatty acid concerned was an acid containing at least 16 carbon atoms, and was quite different from fluoroacetic acid, the toxic principle of *Dichapetalum cymosum*.

Fig. 2. Results of a run of a mixture of 1.05 mg. of 12-fluorododecanoic acid and 2.1 mg. of 18-fluorostearic acid by reversed-phase chromatography. Stationary phase liquid paraffin. Mobile phase starts with 40% (v/v) acetone-water changing to 55 and 60% acetone-water at the points indicated.

SUMMARY

1. The main toxic principle in the seeds of *Dichapetalum toxicarium* behaves as a long-chain fatty acid containing fluorine.

2. Upon injection into rats, or administration in the food, it induced large citric acid accumulations, especially in the heart. The toxicity is therefore due presumably to a conversion into fluorocitric acid.

3. With kidney particles from the guinea pig, the fluoro fatty acid fraction induced citric acid accumulations.

We are indebted to Dr F. L. M. Pattison (London, Ontario) for specimens of synthetic ω -fluorodecanoic, ω -fluorododecanoic and ω -fluorostearic acids. Fluorine analyses were carried out at A.E.R.E., Harwell, by a spectrochemical method.

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Bacterial Degradation of the Nitrobenzoic Acids

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Recent work on the dissimilation of nitrate by micro-organisms and plants suggests that, during reduction to the amino level, nitrite is 'fixed' as an organic nitro derivative (de la Haba, 1950). The existence of enzyme systems capable of metabolizing nitro compounds in liver (Egami & Itahishi, 1951), *Neurospora crassa* (Little, 1951) and pea plants (Little, 1957) lends further evidence for the possibility that nitro compounds are widespread functional intermediates in nitrogen metabolism. In none of the three systems mentioned was there prior contact with environmental nitro compounds, so that adaptation to these materials could not explain the presence of nitro-metabolizing enzymes.

Although nitro compounds, e.g. chloromycetin (Ehrlich, Gottlieb, Burkholder, Anderson &

Pridham, 1948) and β -nitropropionic acid (Bush, Touster & Brockman, 1951) are synthesized by micro-organisms, the dissimilation of such materials by bacteria and fungi to provide the sole source of carbon, nitrogen and energy has not been widely reported. Simpson & Evans (1953) demonstrated the oxidation of nitrophenols to the corresponding dihydric phenols by *Pseudomonas* and the oxidation of oximes to keto acids and nitrite has been shown to occur (Jensen, 1951; Quastel, Scholefield & Stevenson, 1952). Some aspects of the nitrogen metabolism of strains of *Nocardia* utilizing the nitrobenzoic acids were described by Cain (1958), who found ammonia as the principal nitrogenous product. This paper is concerned with the oxidative metabolism of the nitrobenzoates by some strains of *Nocardia*.

EXPERIMENTAL

Organisms. *Nocardia erythropolis*, isolated from *p*-nitrobenzoate enrichments and *N. opaca* from *o*-nitrobenzoate enrichments, were described by Cain (1958). Recently, after attempted enrichment for 2 years, a number of isolates capable of metabolizing the *meta*-isomer of nitrobenzoic acid were obtained. Examination by standard bacteriological procedures showed these to be *Nocardia* species (hereafter referred to as *Nocardia* M1) which grew initially as Gram-positive filaments disintegrating later (3–5 days) into short rod and coccoid elements. Strains of all species were maintained by subculture upon nutrient-agar slopes and in their respective defined media. A Gram-negative rod, *Pseudomonas* L2, was isolated from laboratory air in *p*-aminobenzoate–mineral salts enrichment cultures and found to utilize *p*-aminobenzoic acid as sole source of carbon, nitrogen and energy, degrading it via *p*-hydroxybenzoate and protocatechuate (cf. Durham, 1956). Dried-cell preparations were prepared either by freeze-drying or by slow-drying at 0° in a desiccator.

Media. The defined medium consisted of (g./l.): KH_2PO_4 , 0.1; K_2HPO_4 , 0.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; aromatic substrate, 1.0; trace-elements solution 10 ml./l.; pH 7.2–7.4. Where a supplementary N-source was required, 0.5 g. of $(\text{NH}_4)_2\text{SO}_4$ /l. was added. Large amounts of cells were produced as described by Cain (1958).

Adapted cells. Under growth conditions where the specific substrate was unstable, adapted cells were produced by the 'exposure technique' of Silliker & Rittenberg (1951); otherwise growth upon media containing the specific substrate was adopted. Unadapted cells were grown on a medium consisting of glucose (2%, w/v) and either $(\text{NH}_4)_2\text{SO}_4$ or L-asparagine (0.5%), with the salts described under Media.

Cell-free extracts. Extracts of the organisms were prepared either by grinding with alumina (McIlwain, 1948) or by ultrasonic disintegration. The latter was effected with a chromium-plated brass transforming stub (length 9 cm., 5A; diam. of the upper half 3.7 cm., of the lower 2.0 cm.) attached to the transducer head of the 50W Mullard Ultrasonic Drill. This probe was immersed in a buffer suspension of the cells (approx. 30 ml.; 0.1–0.2 g. dry wt./ml., but widely variable) in a thick-walled glass tube standing in melting ice. At 20 kcy./sec., about 20–30 min. was sufficient to give a 90–95% breakage (as determined originally by electron microscopy) of a suspension of the above consistency. With less dense suspensions, shorter times were sufficient. Cell debris was removed by centrifuging at 4000–6000 *g* for 15 min. and the opalescent supernatant, which gave a marked Tyndall effect, was separated if required into soluble and particulate fractions by centrifuging at 100 000 *g* for 30–40 min. in a Spinco Model L preparative ultracentrifuge.

Estimations. Nitrite was estimated by the Griess-Ilosvay method (Wallace & Neave (1927) with *NN*-dimethyl- α -naphthylamine as the coupling reagent; ammonia by nesslerization, after distillation (from alkaline solutions in Conway units) from the deproteinized [10% (w/v) trichloroacetic acid] supernatants at the end of an experiment; hydroxylamine by the method of Zucker & Nason (1955). Protein was estimated both by the biuret method (Gornall, Bardawill & David, 1949) and by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951)

with Bovine Albumin, fraction V (The Armour Laboratories) as a protein standard. Because the results of these two methods did not exactly agree, a small correction factor was applied when estimations on the same protein solutions were performed by both methods. Keto acids were estimated by the method of Friedmann & Haugen (1943); succinic acid was estimated manometrically at 37° with a succinoxidase preparation from ox-heart muscle (Umbreit, Burris & Stauffer, 1949) after destruction of any malonic acid by oxidation with 3% (w/v) potassium permanganate in acid solution and subsequent extraction of the succinic acid with ether. β -Oxoadipic acid was qualitatively detected by the Rothera reaction (Fearon, 1948) and estimated manometrically by catalytic decarboxylation at pH 4.0 with 4-aminopyridine (Sistrom & Stanier, 1953).

Manometric procedures. Oxygen uptake and carbon dioxide production were followed by the usual manometric method with the Warburg respirometer (Umbreit *et al.* 1949). Manometry was especially convenient for rapid estimation of protocatechuate with cell-free extracts of *N. erythropolis* or *Nocardia* M1 or with the partially purified oxidase from the former (R. B. Cain, unpublished results). Gas exchanges were measured at 30° unless otherwise specified.

Chromatography. Phenolic compounds were run in one, and occasionally two, directions in the following solvent systems (all quantities by vol.): (A) butanol–acetic acid–water (4:1:5); (B) benzene–acetic acid–water (2:2:1); (C) aq. 5% (w/v) sodium formate, containing 0.5% (v/v) of 98% formic acid. The system (D), ethanol–aq. NH_3 soln. (sp.gr. 0.880)–water (80:4:16) (Long, Quagh & Stedman, 1951), was used for aliphatic non-volatile acids but was also found to be an excellent solvent system for phenolic acidic compounds, giving concise, well-separated spots for a large number of mono- and di-hydric phenols and aminohydroxybenzoic acids. Keto acids were run as their 2:4-dinitrophenylhydrazones in (E) *tert*-amyl alcohol–propanol–aq. NH_3 soln. (sp.gr. 0.880) (65:5:30) and in (F) propanol–aq. NH_3 soln. (sp.gr. 0.880)–water (6:3:1). For confirmation of identity, aqueous solutions of the dinitrophenylhydrazones were reduced with hydrogen and Adams catalyst (platinic oxide). When uptake of hydrogen ceased, the catalyst was filtered off, and the volume reduced *in vacuo* and the resulting amino acid solution run in α -picoline saturated with water, and in ethyl acetate–acetic acid–propan-2-ol–water (11:2:1:3) (F. W. Moore, personal communication).

Phenolic compounds were detected by exposure to ultraviolet light, by spraying with aq. 1% (w/v) FeCl_3 soln. or with an alkaline, 1% diazotized solution of I.C.I. 5091 (4-aminophenyl-2'-diethylaminoethyl sulphone; L. Light and Co. Ltd.); aliphatic acids, with a bromothymol blue–aq. NH_3 soln. spray; amino acids, with ninhydrin and heating at 90–100° for 5 min.; unsaturated aliphatic compounds, with a 1% solution of potassium permanganate in water–acetone (50:50, v/v); nitro compounds, by Ehrlich's reagent [1% (w/v) *p*-dimethylaminobenzaldehyde in 200 ml. of butanol–ethanol (30:170, v/v) + conc. HCl (30 ml.)] after reduction to the amino compound by spraying with acidic aq. 0.5% (w/v) SnCl_2 soln. Whatman no. 542 paper was used for all except the amino acid runs; for the latter, Whatman no. 20 paper was used.

Buffers. Incubations were carried out in either Na_2HPO_4 – KH_2PO_4 buffer or in 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer. The proportions of each constituent were varied according to the pH required, and the

molarity of the resulting buffers varied as indicated in the text.

Spectrophotometry. Measurements of ultraviolet-absorption spectra were made with a Unicam SP. 500 instrument at 5 μ intervals, except at the absorption maxima and minima where measurements at 2 μ were made.

Chemicals. All melting points are uncorrected. Most compounds used were commercial samples which were purified by recrystallization and subsequently shown to be chromatographically pure. All reagents were of A.R. grade.

β -Oxadipic acid, m.p. 119°, was synthesized essentially by the method of Bardhan (1936) but with the use of the ethyl rather than the methyl esters, and recrystallized from ethyl acetate and light petroleum (b.p. 60–80°). The 2,4-dinitrophenylhydrazone of laevulinic acid was prepared by addition of a saturated solution of 2,4-dinitrophenylhydrazine in 2*N*-HCl to an aqueous solution of β -oxadipic acid, and the resulting solution boiled to complete the decarboxylation and the precipitate, obtained on cooling, was twice recrystallized from ethyl acetate and light petroleum (b.p. 60–80°) to give a product with m.p. 200°. 4-Nitrocatechol was synthesized from guaiacol by methylation, nitration of the veratrole produced and subsequent demethylation of nitroveratrole with saturated HBr solution, sp.gr. 1.7. The product, precipitated by cooling, was recrystallized from water containing a little dil. HCl and allowed to stand in a desiccator over KOH. The material melted at 172–173°. 3-Nitrocatechol, m.p. 76–78°, was isolated from the products of the nitration of catechol (Weselsky & Benedikt, 1882) by continuous extraction with benzene in a Soxhlet apparatus, and crystallization of the benzene-soluble material from water. 2-Hydroxy-4-nitrobenzoic acid (*p*-nitrosalicylic acid) and the corresponding *o*-aminosalicylic acid were synthesized by the method of McGhie, Morton, Reynolds & Spence (1949); 3-hydroxy-4-nitrobenzoic acid by the method of Brenans & Prost (1924) and the amino compound by reduction methods similar to those used for the 2-hydroxy isomer; 4-hydroxylaminobenzoic acid, by the method of Bauer & Rosenthal (1944). 4-Nitrosobenzoic acid was prepared by oxidation of the hydroxylamino compound with excess of FeCl_3 . The nitrosobenzoic acid, after initial precipitation, was soluble with difficulty in ethanol and separated from hot solutions of this solvent as an amorphous yellow powder. This material was dissolved in ethanol containing a small quantity of ammonia and applied to a column (1 cm. $\times$ 15 cm.) of Whatman cellulose powder, which was developed with solvent *D*. Whereas the original material gave six or seven spots in ultraviolet light and with SnCl_2 and Ehrlich sprays when run on paper in the same solvent system, the product obtained as a pale-green, single, distinct band from the column with about 100 ml. of solvent gave only three such spots. This material was applied as a strip to the top of sheets of Whatman no. 542 paper and run overnight in solvent *D*. The material corresponding to nitrosobenzoic acid, identified by the diphenylamine–conc. H_2SO_4 test, was cut out, eluted by continuous extraction with ammoniacal ethanol and precipitated by reducing the volume *in vacuo* and acidifying. *p*-Nitrosobenzoic acid gave an intense crimson colour by the diphenylamine–conc. H_2SO_4 test and a pale-green solution with ammonia. Neither 4-nitrosobenzoic acid nor 4-hydroxylaminobenzoic acid melted up to 300°. Hydroxyquinol (1:2:4-trihydroxybenzene) was synthesized by the method of Vliet (1925).

RESULTS

Oxidation of the nitrobenzoate substrates

Washed-cell suspensions of the *Nocardia* isolates oxidized their respective substrates without a lag and with a Q_{O_2} range of 3.5–8.0 for *p*-nitrobenzoate, 12.0–18.5 for *o*-nitrobenzoate and 7.0–11.5 for *m*-nitrobenzoate, the average values being 7.0, 14.0 and 10.0 respectively. Uptake of oxygen, output of carbon dioxide and production of either ammonia or nitrite proceeded at constant rate until the substrate was exhausted, as determined by chromatography, and then fell sharply. The optimum pH for the oxidation of the nitrobenzoic acids by washed cells was 6.0–6.3. This contrasted with the pH for optimum growth, which is appreciably on the alkaline side of neutrality. Below pH 6.8, no growth of any of the isolates occurred on defined medium containing nitrobenzoic acid. This behaviour cannot be accounted for by the greater permeability of the intact cell to the undissociated form of the nitrobenzoate molecules existing at lower pH values, for dried preparations in which Sleeper, Tsuchida & Stanier (1950) first showed the effects of the permeability barrier to be minimal or absent, exhibit the same pH optimum.

The recorded oxygen uptakes and carbon dioxide outputs by washed-cell suspensions of all three species acting on their substrates were usually lower than the theoretical values, although the production of ammonia usually approached the theoretical value. Such discrepancies are not uncommon with the oxidase systems of many micro-organisms (cf. Hughes, 1955; Jayasuriya, 1955; Appleyard & Woods, 1956; Shilo & Stanier, 1957) and probably reflect the occurrence of appreciable oxidative assimilation (see below). The results of some typical experiments are recorded in Table 1. Growth on defined media containing *o*-nitrobenzoate or *p*-nitrobenzoic acid produces much ammonia but only traces of nitrite, whereas with *m*-nitrobenzoic acid, nitrite production predominates (Cain, 1958; D. McKenna, unpublished results); these results are confirmed by the findings shown in Table 1 and suggest the occurrence of the following reactions:

for *p*-nitrobenzoic acid and *o*-nitrobenzoic acid;

for *m*-nitrobenzoic acid.

Action of some inhibitors on the oxidation of p-nitrobenzoic acid

The oxidation rate and total oxygen uptake of *p*-nitrobenzoic acid were consistently stimulated by 0.167 mM-2,4-dinitrophenol (DNP), a well-known

Table 1. *Stoichiometry of the oxidation of the nitrobenzoic acids by species of the genus Nocardia*

Each Warburg flask contained: KOH or H₂SO₄, 0.2 ml.; substrate 1–5 μ moles; 0.1 M-phosphate buffer, pH 7.0, 1.5 ml.; washed-cell suspension, 3.0–21.5 mg. dry wt.; inhibitor to concentration shown. *p*-Nitrobenzoic acid was oxidized by *N. erythropolis*, *o*-nitrobenzoic acid by *N. opaca* and *m*-nitrobenzoic acid by *Nocardia* M1. (DNP, 2,4-dinitrophenol.)

Substrate	Expt. no.	Inhibitor and concn.	Moles/mole of substrate oxidized*			
			O ₂	CO ₂	NH ₃	HNO ₂
<i>p</i> -Nitrobenzoic acid	1	—	3.2	—	0.91	Nil
	2	—	6.1	6.2	0.68	Nil
	3	mm-Isoniazid	3.5	4.2	1.06	Nil
	4	0.167 mm-DNP	5.5	—	1.00	Nil
	5	—	4.1	4.7	0.88	Nil
<i>o</i> -Nitrobenzoic acid	1	—	3.1	3.7	0.82	Nil
	2	—	3.4	—	0.73	Nil
	3	—	3.3	3.6	1.08	Nil
<i>m</i> -Nitrobenzoic acid	1	—	4.3	—	Nil	0.40
	2	—	4.2	3.9	Nil	0.47
	3	—	3.9	4.3	Nil	0.36
Theoretical values	—	Reaction (1)	5.5	7.0	1.0	Nil
	—	Reaction (2)	7.0	7.0	Nil	1.0

* Corrected for results obtained in the absence of substrate.

inhibitor of oxidative phosphorylation. Lardy & Wellman (1952) showed that oxidative phosphorylation processes may act as the limiting factor in respiration. The 'uncoupling' of oxidative phosphorylation by DNP is demonstrated not only by the inability of the organisms to utilize the energy available from catabolic processes (Chance & Williams, 1956), but also by the increased respiration of the DNP-treated cells. The fact that oxygen uptake approached the theoretical value only in the presence of DNP (0.167 mm) suggests the existence of considerable oxidative phosphorylation [oxidative 'assimilation' as Clifton (1946) originally termed it] in *N. erythropolis*. At concentrations above 0.5 mm, 2,4-dinitrophenol showed an increasing inhibitory effect upon the oxidation by the isolates of all three isomers of nitrobenzoic acid; below 0.05 mm it had no effect.

Behrman & Stanier (1957) showed that arsenite inhibited the complete oxidation of nicotinic acid by pseudomonads and led to accumulation of pyruvate. At 5 and 10 mm, potassium arsenite completely inhibited oxidation of *p*-nitrobenzoic acid by *N. erythropolis*, but at mm, although there was no effect on the Q_{O_2} , uptake of oxygen ceased at 3 moles/mole of *p*-nitrobenzoic acid oxidized, and keto acids accumulated in small quantities (well below that required for reaction 4). The principal keto acid was identified chromatographically as pyruvic acid but oxaloacetic acid was also found. The presence of these substances was confirmed by the identification of α -alanine, aspartic acid and β -alanine (the decarboxylation product of aspartic acid) in the reduction products of the dinitrophenylhydrazones.

Sodium malonate (10 mm) also completely inhibited the oxidation of *p*-nitrobenzoate by *N.*

erythropolis. At mm, oxidation occurred but total oxygen uptake was reduced to 2 moles/mole of substrate oxidized (as required by reaction 3) and succinate was detected in the reaction products, although the quantities were again lower than expected (Table 2). These consistently low results probably reflect the existence of alternative metabolic pathways which are not blocked by the inhibitors tried.

Taniguchi, Sato & Egami (1956) found that the nitrite reductase of a coccus which could tolerate high salt concentrations was completely inhibited by 0.1 mm-hydroxylamine and the hydroxylamine reductase of the same organism was also totally inhibited by 0.5 mm-sodium nitrite. The effects of nitrite and hydroxylamine on the oxidation of some probable nitrogen-containing intermediates at these oxidation levels were investigated with suspensions of *N. erythropolis* grown on *p*-nitrobenzoate (Table 3).

The extensive inhibition by nitrite of the oxidation of the hydroxylamino compound compares with its effect upon hydroxylamine reduction. The lack of any inhibition by nitrite of the oxidation of *p*-nitrobenzoate (whereas the hydroxylamino compound was 90 % inhibited) makes it unlikely that *p*-hydroxylaminobenzoate lies on the oxidative degradation pathway of *p*-nitrobenzoate metabolism; if so, *p*-nitrobenzoate oxidation would be comparably inhibited.

Table 2. *Oxidation of p-nitrobenzoate by washed cells of Nocardia erythropolis in the presence of arsenite and malonate*

Each Warburg flask contained: *p*-nitrobenzoate, 2–5 μ moles; KOH, 0.2 ml.; 0.1 M-phosphate buffer, pH 7.2, 1.5 ml.; cell suspension, 7.2–14.0 mg. dry wt./flask over several experiments; inhibitor, to give a final concentration of mM; water, to final vol. 3.0 ml. Reaction was stopped immediately at the cessation of O₂ uptake by addition of 1 ml. of 10% trichloroacetic acid to the flask, and the contents were centrifuged. Analyses were made on the supernatant. Temp. 30°.

Expt. no.	Inhibitor	Moles/mole of <i>p</i> -nitrobenzoate oxidized			
		Succinate	Pyruvate	O ₂	NH ₃
1	None	Nil	Nil	4.1	0.88
2	None	Nil	Nil	3.5	1.06
Theory for reaction (1)	—	Nil	Nil	5.5	1.00
3	mM-K ₃ AsO ₃	Nil	0.1	2.7	0.95
4	mM-K ₃ AsO ₃	Nil	0.2	2.8	1.10
5	mM-K ₃ AsO ₃	Nil	0.2	3.0	0.70
Theory for reaction (2)	—	Nil	1.0	3.0	1.00
6	mM-Sodium malonate	0.3	Nil	2.0	1.04
7	mM-Sodium malonate	0.4	Nil	2.0	1.02
Theory for reaction (3)	—	1.0	Nil	2.0	1.00

Table 3. *Oxidation of p-nitrobenzoic acid and its reduction products by Nocardia erythropolis and the effects of sodium nitrite and hydroxylamine thereon*

Each Warburg flask contained: KOH, 0.2 ml.; 0.1 M-phosphate buffer, pH 7.2; 1.2 ml. of substrate, 2 μ moles; cell suspension, 10 mg. dry wt.; inhibitor, to a final concn. mM (where required); water, to a final vol. 3.0 ml. Oxygen uptake in the inhibition experiments was measured from 30 to 60 min., over which period there was a linear relationship with time. Temp. 30°. Gas phase, air. *N. erythropolis* was grown on *p*-nitrobenzoate.

Substrate	Moles/mole of substrate oxidized (in the absence of inhibitors)		Inhibition of oxidation (%) by	
	O ₂	NH ₃	NaNO ₂	NH ₂ OH
<i>p</i> -Nitrobenzoate	4.5	0.91	50*	100
<i>p</i> -Nitrosobenzoate	3.9	0.72	55	97
<i>p</i> -Hydroxylaminobenzoate	3.4	1.06	90	50
Theoretical NH ₃ production from all substrates	—	1.00	—	—

* Percentage stimulation.

Oxidation of other aromatic compounds by washed suspensions

Several hydroxyaromatic compounds were found to support growth of *N. erythropolis* and *N. opaca* (Cain, 1958), and the ability of washed suspensions of cells of these organisms, grown on *p*-nitrobenzoate and *o*-nitrobenzoate respectively, to oxidize some hydroxyaromatic and other related compounds was determined (Figs. 1, 2). *N. erythropolis* completely oxidized *p*-hydroxybenzoate, protocatechuate, 4-nitroso- and 4-hydroxylamino-benzoate and 4-nitrocatechol rapidly and without a lag. Catechol and benzoate exhibited a short lag but *p*-aminobenzoate, 2- and 3-hydroxy-4-nitrobenzoate and 3-hydroxy-4-aminobenzoate were not oxidized. Low rates of oxygen uptake were shown by quinol, hydroxyquinol, 2:3-, 2:4-, 2:5- and 3:5-dihydroxybenzoate, *p*-aminosalicylate and *m*-nitrobenzoate. *N. opaca* immediately oxidized *o*-nitrobenzoate, catechol and 4-nitrocatechol. Gentisic acid was oxidized at variable rates. Fig. 2 records the highest values obtained, but some pre-

parations of washed cells failed to show activity towards this substrate. Anthranilate, protocatechuate and salicylate were only slowly oxidized at pH 7.0; 3-hydroxyanthranilate, 3-nitrocatechol and several dihydroxybenzoates did not stimulate oxygen uptake. *Nocardia* M1 oxidized only protocatechuate at a high rate.

The application of the principles of simultaneous adaptation (Stanier, 1947) to these results (Fig. 3*a-d*) showed that whereas *p*-nitrobenzoate-grown cells of *N. erythropolis* oxidized 4-nitrocatechol, *p*-nitroso-, *p*-hydroxylamino- and *p*-hydroxy-benzoate and protocatechuate without a lag, cells grown on *p*-hydroxybenzoate oxidized this substrate and protocatechuate; but cells grown on protocatechuate oxidized only protocatechuate, although adaptation to *p*-hydroxybenzoate by these cells was extremely rapid. The adaptation results would suggest that oxidation via *p*-hydroxybenzoate and protocatechuate was occurring with *N. erythropolis* grown on *p*-nitrobenzoate. This oxidation did not proceed via *p*-aminobenzoate as had at first been suspected, for

even after incubation with *p*-aminobenzoate for 24 hr. washed cells failed to show any appreciable oxidative attack on this substrate ($Q_{O_2} < 2.0$), whereas *Pseudomonas* L2, grown on the defined medium + *p*-aminobenzoate, showed a Q_{O_2} of 18.5 towards *p*-aminobenzoate.

The results with washed suspensions of *N. opaca* grown on *o*-nitrobenzoate were far less conclusive. The rates of oxidation of salicylate and several dihydroxy compounds tested were considerably lower than that for *o*-nitrobenzoate itself, although no lag periods were observed. This result could possibly be ascribed to impermeability of the substrate. Although catechol was rapidly oxidized and could reasonably be considered as a potential intermediate, it was the only compound tested which gave a sigmoid curve (Fig. 2); its participation in a degradative pathway was thus suspect. The marked difference in activity towards 3- and 4-nitrocatechol shown by *N. opaca* suggested that the latter has some intermediary position. Cells of *Nocardia* M1 grown on *m*-nitrobenzoate showed an adaptive pattern towards protocatechuate similar to that for

cells grown on *p*-nitrobenzoate; *m*-hydroxybenzoate was not oxidized by washed cells at pH 7.0, although low rates of oxidation were obtained at pH 6.0.

Oxidation by dried-cell preparations

Suspensions of slow-dried and freeze-dried preparations of *N. erythropolis*, grown on *p*-nitrobenzoate, oxidized the same substrates as were oxidized by washed cells, although the Q_{O_2} values were somewhat lower. Dried preparations of cells grown on glucose had no activity, nor did they show any adaptive tendency within 5 hr. Adaptation was not possible in dried cells originally grown on glucose medium, the cells being non-viable (cf. Sleeper *et al.* 1950). The presence of the same pattern of activity in both intact and dried cells grown on *p*-nitrobenzoic acid, and the demonstration that this could not arise by rapid adaptation, considerably strengthens the validity of the suggestions presented by the results with washed cells.

Fig. 1. Oxidation by washed suspensions of *N. erythropolis* of some suspected intermediates in degradation of *p*-nitrobenzoate. Each manometer flask contained: KOH, 0.2 ml.; substrate, 1 μ mole; cell suspension (7.5 mg. dry wt.); 0.067 M-phosphate buffer, pH 7.0, 2.0 ml.; water, to final vol. 3.0 ml. Gas phase, air. ●, *p*-Hydroxybenzoic acid; ○, protocatechuic acid; ▲, catechol; △, *p*-nitrobenzoic acid and 4-nitrocatechol; ▼, *p*-nitrosobenzoic acid and *p*-hydroxylaminobenzoic acid; ○, quinol, hydroxyquinol, 2:4-, 2:5-, 2:6- and 3:5-dihydroxybenzoic acid; ●, endogenous (without added substrate), or with *p*-aminobenzoic acid or 3-hydroxy-4-aminobenzoic acid.

Fig. 2. Oxidation by washed suspensions of *N. opaca* of some possible intermediates in the degradation of *o*-nitrobenzoate. Conditions were as for Fig. 1, but the cell suspension was equivalent to 14.8 mg. dry wt., ○, Catechol; ●, 4-nitrocatechol; △, *o*-nitrobenzoic acid; ▲, gentisic (2:5-dihydroxybenzoic) acid; □, anthranilic or protocatechuic acid; ○, salicylic and 2:6-dihydroxybenzoic acid; ■, endogenous, or with 2:4- or 3:5-dihydroxybenzoic acid or 3-nitrocatechol.

Dried preparations of *N. opaca*, unlike washed suspensions, oxidized salicylate at a significant rate but were also found to possess the ability to oxidize the *para*- and *meta*-isomers of hydroxybenzoic acid. Whereas activity towards 4-nitrocatechol had diminished somewhat, protocatechuate and catechol were rapidly oxidized. Such preparations consistently showed a lag before the maximum rate of oxygen uptake was achieved with *o*-nitrobenzoate (Fig. 4).

Nocardia M1 preparations rapidly oxidized *m*-nitrobenzoate and protocatechuate with the same Q_{O_2} and, in contrast with intact cells, also oxidized *m*-hydroxybenzoate. Dried preparations of cells of *Nocardia* M1 grown on glucose were quite inactive towards these substrates.

Fig. 3. Application of the simultaneous-adaptation techniques to the oxidation of *p*-nitrobenzoic acid by *N. erythropolis*. Each Warburg flask contained: KOH, 0.2 ml.; substrate, 1 μmole; 0.067M-phosphate buffer, pH 7.0, 1.5 ml.; cells equivalent to 6.5 mg. dry wt. standardized turbidimetrically from the 4-day growth harvested from the defined medium + the indicated substrates; water, to final vol. 3.0 ml. Temp. 30°; Gas phase, air. (a) Cells grown with *p*-nitrobenzoate; (b) cells exposed to *p*-aminobenzoate; (c) cells grown with *p*-hydroxybenzoate; (d) cells grown with protocatechuate. Cells exposed to *p*-aminobenzoate were prepared by washing and resuspending the growth from a *p*-nitrobenzoate-mineral salts culture in phosphate buffer and incubating for 24 hr. with 60 μmoles of *p*-aminobenzoic acid at 30°. ●, *p*-Nitrobenzoic acid; ○, *p*-hydroxybenzoic acid; ▲, protocatechuic acid; ■, *p*-aminobenzoic acid; Δ, no substrate.

Intermediary metabolites

Although the application of simultaneous-adaptation methods can give much information on the possible routes of dissimilatory mechanisms, it is open to the objections of impermeability of the cell membranes to polar substrates and of non-specificity of the adaptive response. Confirmation of the presence of intermediates by chemical means is important in formulating a complete pathway.

4-Nitrocatechol. The oxidation of 4-nitrocatechol by preparations of *N. erythropolis* suggests its inclusion on a degradative pathway. Cain (1958) showed that 'catechol substances' were produced in cultures of *N. erythropolis* growing on *p*-nitrobenzoic acid, the maximum concentration occurring about 3-4 days after inoculation. 4-Nitrocatechol was isolated from such cultures as described below.

Three 1 l. cultures, 3 days after inoculation, were centrifuged and the remaining cells removed by Seitz-filtration. The filtrate was acidified with conc. HCl and extracted with ether [6 × 50 ml. or until the aqueous layer no longer gave the Evans (1947) catechol test]. The combined extracts were evaporated to dryness, and the residue was taken up in boiling water (30-40 ml.) and filtered. The filtrate was treated with excess of 20% (w/v) neutral lead acetate and

Fig. 4. Activity of dried-cell preparations of *N. opaca* towards some hydroxy compounds. Conditions were as for Fig. 2, but with cell suspension equivalent to 10 mg. dry wt. and 3 μmoles of substrate. All readings are corrected for endogenous respiration. ●, *o*-Nitrobenzoic acid; □, catechol; ◐, protocatechuic acid; Δ, 4-nitrocatechol; ○, *p*-hydroxybenzoic acid; ×, gentisic acid; ■, salicylic acid; ▲, 2:4- or 3:5-dihydroxybenzoic acid or 3-nitrocatechol.

the small precipitate collected by centrifuging. On allowing to stand overnight at 4°, more precipitate was deposited. The combined precipitates were resuspended in 20 ml. of water and decomposed with H₂S; the PbS was filtered off, washed with hot water and the filtrate and washings were acidified with HCl and ether-extracted. The very small residue (<5 mg.), after removal of the ether, was taken up in ethyl acetate and crystallized by addition of light petroleum (b.p. 60–80°), giving a slightly impure product, m.p. 167° not depressed on admixture with authentic 4-nitrocatechol. Chromatography of the natural product, its mixture with 4-nitrocatechol and authentic 4-nitrocatechol in solvent systems A, B and C respectively gave, in each solvent system, three single spots of identical *R_F* value. The ethanolic solution of the natural product also gave an intense-green with FeCl₃ and a deep-red with NaOH solution, both reactions typical of 4-nitrocatechol.

Monohydroxybenzoic acids. Initially, evidence for the production of *p*-hydroxybenzoate in cultures or in solutions of *p*-nitrobenzoic acid incubated with washed suspensions of *N. erythropolis* was not forthcoming, although the simultaneous-adaptation results strongly suggested that *p*-hydroxybenzoate was an intermediate. Systematic chromatographic examination of the acidic ether-extractable compounds produced by *N. erythropolis* growing on *p*-nitrobenzoic acid, by daily aseptic removal of 2 l. from a 25 l. culture, indicated the presence of a phenolic material with *R_F* identical with that of *p*-hydroxybenzoic acid in solvent systems A, D and F. This appeared after 1 day's incubation, reached its highest concentration at 3 days and had disappeared by the sixth day in vigorously aerated cultures (approx. 10 l. of air/min.). Under conditions of more restricted aeration (1–2 l./min.), where growth was much slower, this phenol was present in quite large amounts at 3–6 days, thereafter slowly disappearing. The eluates of unsprayed areas of paper chromatograms corresponding to the position of *p*-hydroxybenzoic acid gave an ultraviolet spectrum characteristic of this compound (light-absorption max., 245–247 mμ in 0.1 M-phosphate buffer, pH 7.4, changing to 253–255 mμ on addition of HCl to pH 1.0). Growing cultures of *Nocardia* M1 accumulated sufficient *m*-hydroxybenzoate for this compound to be chromatographically detected from quite small volumes of supernatant. This material was isolated in small yield (10 mg.) from 8 l. of culture fluid after 5 days' growth. The natural product, m.p. 200°, gave no melting-point depression on admixture with authentic *m*-hydroxybenzoic acid, m.p. 201°.

Incubation of suspensions of freeze-dried cells of *N. erythropolis* and *Nocardia* M1 with *p*-nitrobenzoate and *m*-nitrobenzoate respectively has demonstrated the production of the *para*- and *meta*-isomers of hydroxybenzoic acid within 6 hr., although with the *para*-isomer other hydroxy

compounds were also present. The accumulation of *p*-hydroxybenzoate was favoured by incubation in tris buffer; in phosphate buffers, little or no hydroxybenzoate was found.

No indication of salicylate has been found in growing cultures or in freeze-dried cells of *N. opaca* incubated with *o*-nitrobenzoate.

Conversion of p-nitrobenzoate, p-hydroxybenzoate and m-hydroxybenzoate into protocatechuate

Although both *p*-hydroxybenzoate and protocatechuate are oxidized by cells of *N. erythropolis* grown on *p*-nitrobenzoate, protocatechuate was never detected in growing cultures. The relative rates of oxidation of the hydroxy compounds and *p*-nitrobenzoate given by manometric experiments suggested that it was unlikely the former would ever accumulate under conditions of good aeration; as Fig. 1 shows, the oxidation of *p*-nitrobenzoate itself is the rate-controlling step. Nevertheless, the fact that dried cells grown on *p*-nitrobenzoate contain enzyme systems active towards both *p*-hydroxybenzoate and protocatechuate and the fact that *p*-hydroxybenzoate is usually oxidized via the dihydroxy compound by *Pseudomonas* (Sleeper & Stanier, 1950) and *Vibrio* O1 (Evans, Smith, Linstead & Elvidge, 1951) invited closer inspection of this stage.

The *Q₀₂* of *p*-nitrobenzoic acid-grown cells with *p*-nitrobenzoate, *p*-hydroxybenzoate and protocatechuate was found to be much lower in tris buffer than in phosphate buffer, although the oxidation still went to completion. Under these conditions and with inadequate aeration, there is a greater possibility of hydroxylated intermediates accumulating. Table 4 shows the results obtained with stationary and agitated suspensions of freeze-dried cells of *N. erythropolis* incubated with *p*-nitrobenzoate and *p*-hydroxybenzoate. Protocatechuate was also detected in a similar manner by ether-extraction of larger volumes (400–500 ml.) of the supernatant solution from similar suspensions incubated with *p*-hydroxybenzoate in phosphate buffer.

The experiment illustrated in Table 4 was repeated on a large scale and the formation of protocatechuic acid was confirmed by its isolation. *p*-Nitrobenzoic acid (2 m-moles) and 2 g. of freeze-dried preparation of *N. erythropolis* in 500 ml. of 0.05 M-tris buffer, pH 7.2, were incubated without agitation at 22–25° for 24–30 hr. At this time, samples of the supernatant gave a pronounced green with FeCl₃; the cells were removed by centrifuging and the supernatant was acidified and continuously extracted with ether for 10 hr. Subsequent treatment was the same as that for 4-nitrocatechol. The isolated material, recrystallized from water, had m.p. 194° and gave no depression on admixture with authentic protocatechuic acid, m.p. 194°. Its ultraviolet spectrum and its chromatographic behaviour in three solvent systems were identical with those of the authentic

material; it was also oxidized by a partially purified protocatechuic acid oxidase from extracts of *N. erythropolis*, with the theoretical uptake of oxygen in manometric experiments. A control experiment with a boiled cell preparation gave no protocatechuate. Protocatechuic acid could also be isolated in considerable quantities from cultures of *N. erythropolis* growing on the defined medium + $(\text{NH}_4)_2\text{SO}_4$ with 0.1% of *p*-hydroxybenzoate as the sole source of carbon. From a 3-day-old 4 l. culture, 0.45 g. was isolated, representing 10% of the theoretical yield.

Similar experiments with *m*-nitrobenzoate and *m*-hydroxybenzoate have also demonstrated the conversion of these substrates into protocatechuate by dried preparations of *Nocardia* M1.

Oxidation of protocatechuic acid

Freeze-dried cells. Freeze-dried preparations of both *N. erythropolis* and *Nocardia* M1, grown on *p*-nitrobenzoate and *m*-nitrobenzoate respectively,

rapidly oxidized protocatechuate. Whereas the oxidation went essentially to completion with washed suspensions, freeze-dried cells took up only 2 atoms of oxygen/mole of protocatechuate oxidized, with the concomitant release of 1 mole of carbon dioxide and the accumulation of a material giving a positive Rothera reaction, which was identified as β -oxoadipic acid. The stoichiometry of these reactions is given in Table 5.

The supernatant at the termination of a protocatechuate oxidation experiment in a Warburg flask was treated with 2 ml. of saturated 2:4-dinitrophenylhydrazine in hydrochloric acid and boiled. The hydrazone was extracted with ether, sodium bicarbonate and, after acidifying, by chloroform (Isherwood & Cruickshank, 1954) and chromatographed with authentic laevulic 2:4-dinitrophenylhydrazone. Both the natural product and the marker gave identical R_f values.

Table 4. *Production of protocatechuate by preparation of Nocardia erythropolis and Nocardia M1*

Each 500 ml. Erlenmeyer flask contained: *p*-nitrobenzoic acid, *p*-hydroxybenzoic acid, *m*-nitrobenzoic acid or *m*-hydroxybenzoic acid, 100 μ moles; 0.05M-tris buffer, pH 7.2, 100 ml.; freeze-dried cell preparation, 100 mg. The control flasks contained a boiled cell preparation. Agitated incubations were made by vigorous shaking on a Microid flask shaker. Anaerobic incubations were performed in sealed vessels, evacuated and flushed with N_2 (O_2 -free). Room temperature ($22 \pm 2^\circ$). *Nocardia* M1 had been grown on *m*-nitrobenzoic acid, *N. erythropolis* on *p*-nitrobenzoic acid. At given times, 25 ml. samples were withdrawn, acidified and extracted with ether. The residue, after removal of ether, was taken up in 1 ml. of ethanol and equal volumes (0.2 ml.) were spotted on to sheets of Whatman no. 542 paper, together with the authentic markers. Replicate sheets were run in each of the butan-1-ol, ethanol and benzene solvents, and sprayed with either FeCl_3 or I.C.I. 5091. Visual estimation was made of the intensities of the spots; these are designated: 0, $\pm$, +, ++, +++, etc.; . = not recorded.

Substrate	Protocatechuate produced				
	Anaerobic 16 hr.	Stationary 16 hr.	Agitated		
			4 hr.	12 hr.	18 hr.
<i>p</i> -Nitrobenzoic acid	+	++++	0	$\pm$	0
<i>p</i> -Hydroxybenzoic acid	+	++++	$\pm$	++	+
<i>p</i> -Hydroxybenzoic acid (phosphate buffer)	.	$\pm$ or 0	0	0	+
<i>m</i> -Nitrobenzoic acid	.	++	.	0	.
<i>m</i> -Hydroxybenzoic acid	.	++	.	0	.

Table 5. *Oxidation of protocatechuate by Nocardia erythropolis*

Each Warburg flask contained: 0.2 ml. of KOH or H_2SO_4 ; substrate, 2–6 μ moles; freeze-dried cell preparation, 10 mg. dry wt.; 0.1M-phosphate buffer, pH 7.0, 1.5 ml.; water, to final vol. 3.0 ml. At the termination of oxidation, flasks were removed, KOH was swabbed out and the contents of the main compartment were made acid, pH 4.0, by the addition of 0.1 ml. of acetic acid; 0.1M-4-aminoantipyrine, 0.4 ml., was added to the side arm; the flasks were then re-equilibrated and the contents of side arm and main compartment mixed. Shaking was continued until gas evolution had ceased. Temp. 30° . *N. erythropolis* had been grown on *p*-nitrobenzoic acid. Essentially the same results were obtained with *Nocardia* M1 preparations.

Proto- catechuate supplied (μ moles)	Substance evolved or consumed (μ l.)			Moles/mole of protocatechuate oxidized		
	O_2	CO_2	β -Oxoadipate	O_2	CO_2	β -Oxoadipate
2.0	46	—	40	1.02	—	0.89
2.5	55	56	51	0.98	1.00	0.91
5.0	110	114	106	0.98	1.02	0.95
6.0	134	133	124	1.00	0.99	0.94
Theory	—	—	—	1.00	1.00	1.00

Table 6. *Oxidation of protocatechuic acid by a desiccator-dried preparation of Nocardia erythropolis*

Each Warburg flask contained: KOH, 0.2 ml.; protocatechuic acid, 2.5 μ moles; 0.067 M-phosphate buffer, pH 7.0, 1.5 ml.; cell preparation, 7.5 mg. dry wt.; inhibitor (when required) to a final concn. of mM; water to total vol. 3.0 ml. Temp. 30°. When O₂ uptake had ceased, the contents of each main compartment were deproteinized with 1 ml. of 10% trichloroacetic acid and continuously ether-extracted for 5 hr. The ether layer was evaporated almost to dryness, a little water added and the remaining ether boiled off. The aqueous layer was made up to 1.0 ml. and succinic acid estimated with an ox-heart preparation (see Experimental).

Expt. no.	Inhibitor	Moles/mole of protocatechuate	
		Uptake of O ₂	Succinate production
1	—	1.94	0
2	mM-Sodium malonate	1.02	1.00
	—	1.91	0
	mM-Sodium malonate	1.20	0.95
	Theory for succinate production (reaction 5)	1.00	1.00
	Theory for fumarate production (reaction 6)	1.50	0
	Theory for oxaloacetate production (reaction 7)	2.00	0

Table 7. *Significance of the oxidative pathway from protocatechuate to β -oxoadipate in Nocardia erythropolis*

Each Warburg flask contained: KOH, 0.2 ml.; sodium protocatechuate, 2 or 10 μ moles; 0.067 M-phosphate buffer, 1.5 ml.; washed cells (suitably diluted) or extract derived from original suspension, 0.5 ml. (equivalent to 10.4 mg. dry wt. of cells and 6.8 mg. of protein in the extract); water to 3.0 ml. Temp. 30°; gas phase, air. The difference of the ratio (3) between A and B is within the limits of experimental error of the manometric technique. Since cell-free extracts of *N. erythropolis* oxidize protocatechuate exclusively to β -oxoadipate and the ratio of (2)/(1) is the same for both intact cells and extract, the results indicate that protocatechuic acid oxidation in intact cells of *N. erythropolis* proceeds entirely by way of β -oxoadipic acid, without the participation of other routes.

	Washed cells (A)	Extract (B)
Total uptake of O ₂ for complete oxidation of 2 μ moles of sodium protocatechuate (μ l.)	156	45
1. Therefore uptake of O ₂ / μ mole of substrate oxidized (μ l.)	78	22.5
2. Rate of uptake of O ₂ (μ l./hr.) in the presence of excess of substrate (10 μ moles)	174	50
3. Ratio of (2)/(1)	2.24	2.22

The identity of the Rothera-positive material with β -oxoadipic acid was confirmed by a large-scale experiment. Protocatechuic acid (1 m-mole) was incubated with 300 mg. of freeze-dried cells of *N. erythropolis* in 300 ml. of 0.1 M-phosphate buffer, pH 7.2, at 30°. At the end of the oxidation, as determined by a pilot experiment in a Warburg flask, half of the supernatant, freed from cell suspension, was acidified with HCl, shaken with excess of 2:4-dinitrophenylhydrazine and heated to complete the decarboxylation. The hydrazone was extracted as before, and the CHCl₃ distilled off and the residue recrystallized from an ethyl acetate–light petroleum (b.p. 40–60°) mixture. The natural derivative, m.p. 197°, gave no melting-point depression on admixture with authentic laevulinic dinitrophenylhydrazone (m.p. 200°). The remain-

ing supernatant was evaporated to a small volume under reduced pressure and exhaustively extracted with ether by shaking (to avoid heating and subsequent decarboxylation); the ether layer was distilled off at low pressure and the residue recrystallized from ethyl acetate and light petroleum. It had m.p. 117° and did not depress that of authentic β -oxoadipic acid, m.p. 119°, on admixture.

Desiccator-dried cells. Preparations of *N. erythropolis* dried in a desiccator showed uptakes of oxygen which varied between 1 and 2 moles/mole of protocatechuate oxidized. The uptake of oxygen was usually nearer 2 moles/mole of protocatechuate, and under these circumstances the Rothera test on the supernatant at the end of the reaction was either negative or very weak. Kilby (1951) and Katagiri & Hayaishi (1957) showed that β -oxoadipic acid was further metabolized to succinate; assuming that oxidation was proceeding through β -oxoadipate in this organism, succinate and other terminal oxidation products should result. One particular batch of desiccator-dried cells consumed exactly 2 moles of oxygen/mole of protocatechuate and was tested for succinate production by inhibition with malonate. Under these conditions, succinate accumulated and estimation of succinate, after malonate had been destroyed, gave the theoretical value (Table 6). Chromatography of the supernatants from the experiments where malonate had been present showed the presence of succinate only, whereas in the absence of the inhibitor small quantities of fumarate and malate were identified. No oxaloacetic acid, which is required as the product of reaction (7), was found. With most batches of desiccator-dried cells, however, the quantitative data were less definite.

Cell-free extracts. Extracts of *Nocardia* M1 and *N. erythropolis* (grown on *m*-nitrobenzoic acid and *p*-nitrobenzoic acid respectively) prepared by ultrasonic disintegration or by grinding with alumina, oxidized protocatechuate to β -oxoadipate with the same stoicheiometry as freeze-dried cells. Table 7 shows the degree of protocatechuate oxidation by washed cells of *N. erythropolis* via β -oxoadipic acid. Extracts of *N. opaca* prepared in phosphate or tris buffers also oxidized protocatechuate to β -oxoadipic acid, but were inactive towards both catechol and gentisic acid, even in the presence of Fe^{2+} ions or other bivalent metal ions or glutathione. This pattern of activity was demonstrated for several batches of cells grown on *o*-nitrobenzoic acid. Unexpectedly, and in marked contrast to the results with *N. erythropolis*, protocatechuic acid oxidase activity was also shown by extracts of *N. opaca* grown on the glucose + ammonium sulphate medium from an inoculum which had been carried on nutrient-agar slopes for at least 6 months.

Stanier (1950), using water extracts of dried cells of *Pseudomonas fluorescens* grown on *p*-hydroxybenzoate, found that these extracts oxidized protocatechuate to β -oxoadipate (see Fig. 5) with an R.Q. of 1.0, but on ageing or dialysis against water

the enzymes catalysing loss of carbon dioxide were destroyed and the R.Q. fell to 0.09. This contrasted with an extract of *Neurospora* mycelium where prolonged dialysis had no effect on the decarboxylase activity (Gross, Gafford & Tatum, 1956).

All the protocatechuic acid oxidase activity of *N. erythropolis* was in the soluble-protein fraction after ultracentrifuging. Freshly prepared extracts had an R.Q. of 1.0 but dialysis against distilled water for 40 hr. at 4° reduced the R.Q. to 0.6. After dialysis for 5 days, the R.Q. had fallen further to 0.55 only, which suggests that the stability of the decarboxylase-enzyme complex of *N. erythropolis* is appreciably greater than that recorded for *Pseudomonas*, but resembles that of *Neurospora*. Oxidase and decarboxylase activities of *N. erythropolis* could be completely separated by ammonium sulphate fractionation, all the oxidase activity lying in the fraction precipitated between 0.33 and 0.45 saturation, and the decarboxylase activity in that between 0.5 and 1.0 saturation. The end product of the action of the oxidase on protocatechuate was β -carboxymuconic acid, which was isolated as the sodium salt from a large-scale oxidation with a partially purified oxidase, essentially by the method of McDonald, Stanier & Ingraham (1954), and gave spectral and chromato-

Fig. 5. Metabolic pathways of degradation of nitrobenzoate by species of the genus *Nocardia*.

graphic characteristics identical with those of authentic specimens kindly provided by Dr R. Y. Stanier and Dr S. Gross. No activity towards protocatechuate was found in extracts of either *Nocardia* M1 or *N. erythropolis* after growth on a glucose medium.

DISCUSSION

The utilization of nitro nitrogen as the sole source of this element is rare even among micro-organisms, but it is interesting to observe that the results obtained here for the isomers of nitrobenzoic acid indicate that the nitrogen appears in forms which in nature are more common, namely ammonia and nitrite. Although Simpson & Evans (1953) and Gundersen & Jensen (1956) found that nitrite was produced from nitrophenols by bacteria, *Nocardia* M1 is unusual among the *Nocardia* spp. studied here in being the only organism isolated which liberated nitrobenzoate nitrogen as nitrite. With washed cells of *N. opaca* and *N. erythropolis*, ammonia was the sole product. The appearance of trace amounts of nitrite in growing cultures of *N. erythropolis* and *N. opaca* (Cain, 1958) could be explained by the further oxidation of metabolically produced ammonia to nitrite, although the presence of hydroxylamine has never been convincingly demonstrated.

An alternative explanation is that the nitrogenous product of all the nitrobenzoates is nitrite but that *Nocardia* M1 is unique among the isolates in that it does not possess a nitrite-reductase system yielding ammonia. The fact that *N. erythropolis*, grown on *p*-nitrobenzoate (but not on a glucose-ammonium sulphate medium), does indeed possess nitrite-reductase and hydroxylamine-reductase systems, whereas these activities are absent in *Nocardia* M1 (R. B. Cain, unpublished results) and the observation that nitrite stimulated the '*p*-nitrobenzoate-oxidase' complex in *N. erythropolis*, suggest that this explanation is more likely. Absolute confirmation can be obtained only by following nitrite accumulation from *p*-nitrobenzoate in the presence of inhibitors of the nitrite reductase; this has not yet been achieved without arresting the oxidation of *p*-nitrobenzoate itself. Although no specific investigations have been carried out with *N. opaca*, the observation that ammonia is the nitrogenous end product of the oxidation of *o*-nitrobenzoate suggests that a nitrite reductase is present in this organism. The complete inhibition of the oxidation of all the nitrobenzoates by hydroxylamine does not detract from the general hypothesis, for at lower concentrations than that used in these experiments (mM) hydroxylamine is a powerful inhibitor of many systems, including the nitrite reductase of a coccus (Tani-

guchi *et al.* 1956). The major objections to the hypothesis are provided by the facts that the oxygen uptake of *p*-nitrobenzoate, even in the presence of dinitrophenol, never exceeded 5½ moles of oxygen/mole of *p*-nitrobenzoate and that neither nitrite nor hydroxylamine has ever been detected either during or at the end of an oxidation with washed cells. Nevertheless, the appearance of nitrogen at the amino level in the *ortho*- and *para*-isomers followed by the incorporation of the free ammonia (Cain, 1958) makes these *Nocardia* species independent of other exogenous sources of nitrogen and provides a means whereby the toxic nitro group can be rendered biologically inert.

The immediate oxidation, at approximately the same rates, of *p*-nitroso- and hydroxylaminobenzoic acid and the similar ammonia production per mole of substrate oxidized, suggested the possibility that these compounds might be the immediate degradation products of *p*-nitrobenzoate. Nitrite (mM) strongly inhibits the oxidation of these materials, in contrast with its stimulatory effect upon *p*-nitrobenzoate oxidation by washed cells, and no evidence for the production of *p*-hydroxybenzoate from either *p*-nitroso- or *p*-hydroxylaminobenzoate has so far been found; any suggestion of their intermediary position on the oxidative pathway is thus tentative.

This is apparently the first time that a protocatechuic acid oxidase has been found in members of the genus *Nocardia*, or has been induced by growth upon an aromatic nitro compound. That such nitro compounds are oxidized by way of hydroxylated intermediates now seems to be well-founded, both for nitrobenzoates and nitrophenols (Simpson & Evans, 1953), and attention should be drawn to the fact that the oxidative pathways for the hydroxy derivatives resemble those found in *Pseudomonas* (Sleeper & Stanier, 1950; Dagley & Patel, 1957), *Vibrio* O1 (Evans, 1947), *Neurospora* (Gross *et al.* 1956), *Hormodendrum* and *Penicillium* (Henderson, 1957) in which hydroxylation in the *ortho*-position to an existing hydroxyl group precedes ring cleavage. Furthermore, the primary products of ring opening are similar to those of *Pseudomonas* (McDonald *et al.* 1954). On the basis of our results, the scheme for oxidative metabolism of the nitrobenzoates shown in Fig. 5 is suggested.

Because of the present conflicting evidence, a pathway for the *ortho*-isomer cannot yet be formulated. The finding that protocatechuic acid oxidase activity exists in cells of *N. opaca* grown on glucose shows that the activity is not stimulated specifically by growth of the cells on *o*-nitrobenzoate, although this appears to be true for both the *para*- and *meta*-isomers. The presence of this enzyme in glucose-grown cells suggests that protocatechuate may be an end product of some metabolic reaction normally

occurring in *N. opaca*. The alternative explanation, that the enzyme is induced by compounds produced during glucose metabolism which structurally resemble protocatechuate, is less likely in view of the extreme specificity of protocatechuic acid oxidase (Stanier & Ingraham, 1954). Gross *et al.* (1956) obtained comparable results when they found β -carboxymuconic acid-lactonizing and -delactonizing activity in glucose-grown cells of *Neurospora*. Although these results do not invalidate the possibility of the oxidation of *o*-nitrobenzoate to protocatechuate, the latter has not, as yet, been conclusively demonstrated as an intermediate in the degradation of *o*-nitrobenzoate.

4-Nitrocatechol is a new bacterial metabolic product, although Robinson, Smith & Williams (1951) found that 0.5% of nitrobenzene administered to rabbits appeared as 4-nitrocatechol. The production of 4-nitrocatechol from *p*-nitrobenzoic acid by *N. erythropolis* is analogous to the oxidation of benzoic acid via catechol by *Vibrio* O1 (Evans, 1947). Such catechols have been considered to arise by dehydrogenation from the corresponding diols, which are produced by 'perhydroxylation' of the ring (Stanier, 1948; Boyland, 1950), and are usually further degraded by dehydrogenation to the corresponding quinone (Evans, 1947; Nelson & Dawson, 1944). Marked dehydrogenase activity towards 4-nitrocatechol, demonstrated by the usual Thunberg techniques, has indeed been observed with washed suspensions of *N. erythropolis* grown on *p*-nitrobenzoate. The isolation of a nitroquinone, however, is likely to prove somewhat difficult in view of the reactivity of the molecule, although the production of intense reddish-brown coloration in media containing *p*- and *o*-nitrobenzoate during growth makes this a not unlikely intermediate product, which would undergo condensations similar to those involved in melanin formation.

SUMMARY

1. *Nocardia erythropolis* and *N. opaca* oxidize *p*-nitrobenzoate and *o*-nitrobenzoate respectively to carbon dioxide and ammonia; *Nocardia* M1 produces nitrite but no ammonia from the *meta*-isomer. The effects of some inhibitors on these oxidations are described.

2. *Nocardia erythropolis* and *Nocardia* M1 convert their respective nitrobenzoate substrate into the corresponding hydroxy compound and thence to protocatechuate. *N. erythropolis* also produces 4-nitrocatechol from *p*-nitrobenzoate.

3. *Nocardia erythropolis* and *Nocardia* M1 grown upon *p*- and *m*-nitrobenzoate respectively, but not upon glucose or nutrient broth, possess the enzyme systems for the oxidation of protocatechuate. Freeze-dried cells and crude extracts convert proto-

catechuate quantitatively into β -oxoadipic acid. Certain desiccator-dried preparations oxidize this keto acid, the more active ones finally yielding succinate.

4. All the protocatechuic acid oxidase activity is in the soluble-protein fraction of crude extracts and can be separated from decarboxylase activity by fractionation with ammonium sulphate, the oxidase activity lying in the fraction precipitating between 0.33 and 0.45 saturation. The end product of partially purified oxidase is β -carboxymuconic acid.

5. Dried cells of *N. opaca* oxidize *o*-nitrobenzoate catechol and protocatechuate rapidly.

6. The possible explanations for the differences in nitrogenous end products are discussed and attention is drawn to the analogous reactions performed by *Nocardia* spp. grown on the nitrobenzoates and by other micro-organisms grown on a variety of ring compounds.

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Enzymes of *Trichomonas foetus*

THE ACTION OF CELL-FREE EXTRACTS ON BLOOD-GROUP SUBSTANCES AND LOW-MOLECULAR-WEIGHT GLYCOSIDES

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Enzymes which destroy the serological activity of the water-soluble blood-group substances have been isolated from many sources (see Kabat, 1956). In those instances where chemical studies have been made the loss of serological specificity was accompanied by the liberation of reducing sugars, indicating that the enzymes are breaking down the carbohydrate portion of the mucopolysaccharide molecules. Thus enzyme preparations from snail (*Busycon*)-liver extract liberate methylpentose and *N*-acetylhexosamine from hog A-substance (Howe & Kabat, 1953) and preparations from *Clostridium welchii* set free fucose, *N*-acetylhexosamine and galactose from hog and human H-substances (Crumpton & Morgan, 1953; Buchanan, Crumpton & Morgan, 1957). György, Rose & Springer (1954) similarly showed that an enzyme preparation from *Lactobacillus bifidus* var. *Penn.* liberates fucose, *N*-acetylhexosamine and galactose from blood-group substances prepared from ovarian cysts and

meconium of all A, B, O blood groups. An interpretation of the changes in the structure of the blood-group mucopolysaccharides brought about by these enzymes is rendered difficult by the extent of disruption of the molecules and the similarity of the enzymic hydrolysis products irrespective of the blood group of the individual from whom the starting material was derived. It would appear that the enzyme preparations contain a mixture of enzymes: hexosaminidases, fucosidases, galactosidases and possibly peptidases, which bring about further breakdown of the molecule after the initial change, which leads to loss of specificity, has occurred. The presence of *N*-acetyl- α - and - β -glucosaminidases in the snail-liver preparations used by Howe & Kabat (1953) has been demonstrated and the preparations from *L. bifidus* var. *Penn.* were known to contain an *N*-acetyl- β -glucosaminidase and a β -galactosidase (György *et al.* 1954), but in no instance was the specificity of the