Bacterial Degradation of the Nitrobenzoic Acids

2. REDUCTION OF THE NITRO GROUP*

By N. J. CARTWRIGHT and R. B. CAIN†
Department of Bacteriology, University of Birmingham

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Reduction of the aryl nitro group by animal tissues (Beuding & Jolliffe, 1946; Parker, 1952), moulds (Zucker & Nason, 1955) and bacteria (Smith & Worrel, 1950; Egami, Ebata & Sato, 1951; Yamashina, 1954; Saz & Slie, 1954*a*, *b*) has been observed. The fundamental importance of this reaction is emphasized by the work of de la Haba (1950) and McElroy & Spencer (1956), from which the suggestion arose that nitro compounds are probably intermediates in the reduction of nitrates by green plants and micro-organisms, and the finding that moulds and higher plants, in the absence of added nitro compounds or their presumed precursors, elaborate enzymes capable of metabolizing nitro compounds (Bush, Touster & Brockman, 1951; Shimoda, 1951; Raistrick & Stössl, 1958; Little, 1957).

The appearance of arylamines in cultures of *Nocardia* species growing on nitrobenzoic acids was noted by Cain (1958*a*), but Cartwright & Cain (1959) found that these compounds appeared to have little significance in the oxidative breakdown of *p*- and *m*-nitrobenzoates, although this is less certain with the *ortho*-isomer.

The study of the reduction of nitrobenzoates by *Nocardia* species and a strain of *Pseudomonas fluorescens* described in this paper includes investigations with cell-free extracts and the stimulation of a nitroreductase by flavin adenine dinucleotide, an effect which had previously been observed only in *Escherichia coli* among the bacteria (Saz & Martinez, 1956).

EXPERIMENTAL

Organism

The organisms used were strains of *Nocardia erythropolis*, *N. opaca*, *Nocardia* M 1 and *Pseudomonas fluorescens* isolated by enrichment methods and grown in bulk on a chemically defined medium by the techniques described by Cain (1958*a*). The procedures for obtaining adapted cells, washed suspensions and cell-free extracts were those described by Cartwright & Cain (1959). The particular strain of organisms used is named in the description of each experiment. Absence of contamination was checked by examination of stained smears and plating of samples.

Chromatography

The solvent systems used were (all quantities by vol.): (A) butanol–acetic acid–water (4:1:5); (B) ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (40:1:6). Arylamino compounds were detected by Ehrlich's reagent or by diazotization and reaction with *N*-naphthylethylenediamine. Phenolic compounds were detected by exposure to ultraviolet light, spraying with $FeCl_3$ or with an alkaline 1% solution of diazotized I.C.I. 5091 (4-aminophenyl-2′-diethylaminoethylsulphone; L. Light and Co. Ltd.).

Incubations

Unless otherwise stated, incubations were performed in Warburg flasks (about 16 ml. volume) attached to manometers which were left open to air. Temperature was maintained at 30° during shaking at 120 strokes/min.

Estimations

Arylamines. The procedure was essentially that of Bratton & Marshall (1939) as modified by Glazko, Wolf & Dill (1949). Calibration curves were prepared by the use of known compounds. Under the conditions used, the coloured complexes of *o*, *m*- and *p*-aminobenzoic acid showed molar-extinction coefficients of 7700, 10 960 and 12 510, respectively. The aminobenzoic acids have linear responses over the range 0–5 μg./ml. (10 μg./ml. with the *ortho*-isomer) and 0·3 μg./ml. of each could be accurately estimated. Anthranilic acid had a lower extinction with consequent less accurate analytical results at low concentrations. For very small quantities of *p*-aminobenzoic acid (less than 0·05 μg./ml.) a microbiological assay was used.

Protein. This was estimated either by the Folin method of Lowry, Rosebrough, Farr & Randall (1951) or by the biuret method of Gornall, Bardawill & David (1949).

Growth. The growth of the *Nocardia* species was estimated turbidimetrically at 530 mμ with the precautions mentioned by Cain (1958*a*).

Ultraviolet spectra. These were plotted from readings on a Unicam SP 500 instrument.

Microbiological assay

Medium. Cutts & Rainbow (1950) described a yeast, *Saccharomyces cerevisiae* 47, having an absolute requirement for *p*-aminobenzoic acid in a synthetic medium supplemented with other growth factors. A culture of this yeast was used to identify *p*-aminobenzoic acid in *p*-nitrobenzoate

* Part 1: Cartwright & Cain (1959).

† Present address: University of Leeds, Department of Biochemistry, 9 Hyde Terrace, Leeds, 2.

cultures by measuring, turbidimetrically, growth of the yeast on a chemically defined medium supplemented with either authentic *p*-aminobenzoic acid or the filtered culture fluid produced by growth of *N. erythropolis* on *p*-nitrobenzoic acid. The medium for yeast growth, which was adjusted to pH 5·0–5·2 and sterilized by autoclaving at 15 lb./in.^{2} for 15 min., contained (in 1 l.): glucose, 40 g.; $(NH_4)_2HPO_4$, 4 g.; KH_2PO_4, 0·2 g.; $MgSO_4,7H_2O$, 0·1 g.; $CaCl_2,6H_2O$, 0·25 g.; lactic acid, 3 g.; 50% (w/v) potassium lactate solution, 12 ml.; trace element solution (Barnett & Ingram, 1955), 10 ml. The vitamin supplement, sterilized by filtration, was added aseptically to the cooled medium to give the following final concentrations (μg./l.): *meso*-inositol, 10 000; (+)-biotin, 2; calcium pantothenate, 1000; thiamine HCl, 1000; pyridoxine hydrochloride, 1000; nicotinic acid, 1000; riboflavin, 50; *p*-aminobenzoic acid, 100.

Assay procedure. The *p*-aminobenzoic acid requirement of yeast 47 was replaced by L-histidine (10 mg./ml.), DL-methionine (20 mg./ml.) and adenine (10 mg./ml.) (Dr C. Rainbow, personal communication), and the sensitivity of yeast 47 to *p*-aminobenzoic acid was markedly increased if the inocula for new cultures were grown for two or three subcultures on a medium devoid of *p*-aminobenzoic acid but supplemented with these factors.

The arylamine produced by incubation of washed cells of *Nocardia erythropolis* with *p*-nitrobenzoic acid was assayed for *p*-aminobenzoic acid after removal of the cells by centrifuging. Samples of this supernatant, together with standards of authentic *p*-aminobenzoic acid of the same concentration (as measured colorimetrically), were sterilized by filtration through a sintered-glass filter (pore size 0·95 μ) before addition to tubes of the previously sterilized medium. The final volume in each tube was 5 ml. and quadruplicate sets of unknown, standard and control tubes were inoculated with 0·05 ml. of a washed suspension of yeast 47. The inoculum culture was grown for 3 days on the *p*-aminobenzoic acid-deficient medium supplemented with adenine, methionine and histidine. It was harvested and washed aseptically, and resuspended to 5 ml. in sterile water. The inoculated test cultures were incubated at 30° for 3 days and the extinction was then read on a Unicam SP. 350 D.G. spectrophotometer at 445 mμ in 10 mm. light-path glass cells.

Chemicals

Most chemicals were commercial products which were twice recrystallized in this Laboratory. *p*-Hydroxylaminobenzoic acid was synthesized by the method of Bauer & Rosenthal (1944), and repeatedly recrystallized till free of *p*-aminobenzoic acid as revealed by chromatography. It did not melt below 300°. *p*-Nitrosobenzoic acid was prepared and purified by the method of Cartwright & Cain (1959), the product agreeing in properties with those given for this isomer by Alway (1904); 2-hydroxy-4-nitrobenzoic acid (*p*-nitrosalicylic acid) and *p*-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 corresponding amino compounds by methods essentially the same as those used to reduce *p*-nitrosalicylic acid. Oxidized and reduced di- and tri-phosphopyridine nucleotides (DPN, DPNH, TPN) were obtained from L. Light and Co. Ltd. and were 95% pure on spectrophotometric assay. Flavinadenine dinucleotide (FAD) (80%) and riboflavin 5-phosphate (FMN) were products of the Sigma Chemical Co., St Louis, Mo., U.S.A.) Adenosine di- and tri-phosphate (ADP and ATP, as the sodium salts) were not assayed but were the products as obtained from L. Light and Co. Ltd.

Buffers

Sørensen (Na_2HPO_4–KH_2PO_4) phosphate buffers and 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffers were used. Concentrations used are mentioned in the text.

RESULTS

Arylamine production by washed suspensions of Nocardia erythropolis *CA* 4 *and* Pseudomonas fluorescens *NC* 3

A correlation between the *p*-nitrobenzoic acid added to the medium and the maximum amount of arylamine produced by *N. erythropolis* in growing cultures was first observed by Cain (1958*a*). Washed suspensions of this organism and *P. fluorescens* also reduced *p*-nitrobenzoic acid, and *N. opaca* and *Nocardia* M 1 reduced *o*- and *m*-nitrobenzoic acid respectively under similar conditions.

The ability to form arylamine from *p*-nitrobenzoic acid was examined with suspensions of *N. erythropolis* grown on *p*-nitrobenzoic acid (adapted) or on

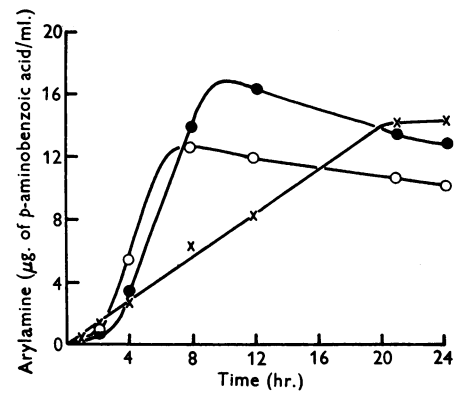

Fig. 1. Arylamine production by washed suspensions of *Nocardia erythropolis* and *Pseudomonas fluorescens*. The incubation mixtures contained: sodium *p*-nitrobenzoate, 1 m-mole; 0·067 M-phosphate buffer, pH 7·4, 200 ml.; cell suspension (*N. erythropolis*, 600 mg. dry wt.; *P. fluorescens*, 450 mg. dry wt.). Final volume, 250 ml. Adapted cells were harvested from defined medium + *p*-nitrobenzoate after growth for 6 days; unadapted cells were harvested from glucose + $(NH_4)_2SO_4$ medium solidified with agar. Portions (3 ml.) were removed at intervals, the reaction was stopped by the addition of 0·5 ml. of 10% (w/v) trichloroacetic acid and estimated for arylamine. Incubations were performed at 30° in 1 l. Erlenmeyer flasks loosely plugged with cotton wool and agitated vigorously on a Microid flask shaker (approx. 300 oscillations/min.; 5 cm. throw). *p*-Aminobenzoic acid: 1 μg./ml. is equivalent to 7·25 μm-moles/ml. ●, *N. erythropolis*, adapted cells; ×, *N. erythropolis*, unadapted cells; ○, *P. fluorescens*, adapted cells.

glucose and $(NH_4)_2SO_4$ (unadapted) and also with suspensions of *P. fluorescens* grown on *p*-nitrobenzoic acid. When incubated with *p*-nitrobenzoic acid in phosphate buffer adapted cells of both organisms reduced the nitro group, with a maximum level of arylamine appearing in 8–10 hr. and then decreasing slowly (Fig. 1). With unadapted cells, the rate of arylamine production was slower, but the maximum level eventually reached was comparable with that from adapted cells. There was no appreciable increase in cell mass or any contamination over this period of time. Suspensions of non-viable dried cells of *N. erythropolis*, grown on *p*-nitrobenzoic acid, also performed this reduction and, in addition, reduced 3-hydroxy-4-nitrobenzoic acid to the corresponding amino compound. Similar preparations of *N. opaca* grown with *o*-nitrobenzoic acid reduced 3-hydroxy-2-nitrobenzoic acid to 3-hydroxyanthranilic acid.

Identification of p-*aminobenzoic acid*

Spectrophotometric identification. At pH 3·5, *p*-aminobenzoic acid has a sharp peak at 225 mμ, which coincides with a minimum in the *p*-nitrobenzoic spectrum. Cell-free filtrates from 7-day growing cultures and from incubations of washed cell suspensions with *p*-nitrobenzoic acid and buffer, when diluted and adjusted to pH 3·5, also showed a sharp peak at 225 mμ, suggesting that *p*-aminobenzoic acid was present in significant quantities. This was confirmed by its isolation from such filtrates.

Chromatographic identification. The acidified supernatants from cultures and from all suspensions similar to those described above were extracted continuously with ether for 30–40 hr.; samples of the concentrated extracts were applied to papers together with authentic *p*-aminobenzoic acid and run in the solvent mixtures A and B. In each solvent system spots moving with the same R_F and yielding the same colour reactions as authentic *p*-aminobenzoic acid were obtained. The naturally produced arylamine failed to separate from authentic material when both were applied in admixture for chromatography.

Microbiological identification. The usefulness of the microbiological method for identifying *p*-aminobenzoic acid in cultures of *N. erythropolis* grown with *p*-nitrobenzoic acid as the substrate was dependent upon the lack of any inhibitory effect against yeast 47 of *p*-nitrobenzoic acid (or other metabolic products) remaining in such cultures. Results obtained on the chemically defined medium with the vitamin supplement containing authentic *p*-aminobenzoic acid (100 μg./l.) showed that *p*-nitrobenzoic acid, up to 0·02 M, produced no diminution of growth. Our inositol sample (from vegetable sources), a possible source of extraneous *p*-aminobenzoic acid, was also shown microbiologically to contain no *p*-aminobenzoic acid. Table 1 demonstrates the identity with *p*-aminobenzoic acid of the arylamine produced by *N. erythropolis* from *p*-nitrobenzoic acid.

Chemical isolation of p-*aminobenzoic acid.* This was undertaken to give final proof of the identity of the arylamine. A filtered *p*-nitrobenzoic acid culture medium (8 l.) after growth of *N. erythropolis*, which showed by colorimetric analysis a total arylamine content of 65 mg., was evaporated *in vacuo* to 300 ml. and, after acidification and removal of the precipitate by filtration, the volume was further reduced to 50 ml. Addition of ethanol (100 ml.) precipitated much inorganic material, which was filtered off. Further evaporation to 25 ml. was followed by acidification with dil. HCl and two extractions with ether to remove the bulk of the reddish-brown pigment. The aqueous layer was neutralized with dil. NaOH and propan-2-ol added to 80% (v/v) to precipitate most of the NaCl. The filtrate was concentrated to about 20 ml., acetic acid (2 ml.) added and the solution continuously extracted with ether for 4 days or until the arylamine content of the aqueous phase was minimal. After evaporation of the ether, the residue was dissolved in water (5 ml.) and applied to a column (1 cm. × 15 cm.) of Amberlite IR-120 (H) resin (Chromatography Grade), which was then washed with water (200 ml.) and 1 mN-acetic acid (50 ml.), whereupon some contaminating brown pigment was eluted. The *p*-aminobenzoic acid fraction was eluted with 100–150 ml. of 2 N-acetic acid. The acidic eluate was extracted continuously with ether for 40 hr., evaporated to dryness *in vacuo* to remove acetic acid and the ether-soluble residual material recrystallized (charcoal) from water (2 ml.) to give fawn-coloured needles (12 mg., m.p. 174–175°). This substance did not depress the m.p. of authentic *p*-aminobenzoic acid; it supported the growth of

Table 1. *Activity of the arylamine formed by reduction of* p-*nitrobenzoic acid for the* p-*aminobenzoate-requiring yeast* 47

Quadruplicate tubes containing the *p*-aminobenzoate-deficient basal medium with the additions shown were inoculated with yeast 47 and incubated for 64 hr. at 30°. Details are given in the Experimental section.

Additions to medium	Final concn. in tubes (μg./ml.)	Growth (E 445 mμ) Mean	Range
p-Nitrobenzoic acid	16·70	0·011	0·006–0·015
p-Aminobenzoic acid	0·027	0·630	0·620–0·640
Arylamine* from supernatant	0·027	0·624	0·618–0·638
None	—	0·018	0·011–0·026

* Estimated colorimetrically as *p*-aminobenzoic acid.

yeast 47 and behaved identically with *p*-aminobenzoic acid on chromatograms. Further proof of the identity of the material was obtained from conversion into the *N*-acetyl derivative by treating with excess of acetic anhydride and sodium acetate on a steam bath for 15 min. The product, isolated by dilution with water and extraction into ether, crystallized from water to form white needles, m.p. 247–248°, undepressed on admixture with *N*-acetyl-*p*-aminobenzoic acid. (All melting points are uncorrected.)

Identification of anthranilic acid

Anthranilic acid was identified from cultures of *N. opaca* grown on *o*-nitrobenzoate (*a*) spectrophotometrically by its absorption max. at 310 mμ, pH 7·0, (*b*) chromatographically by R_F and its violet–blue fluorescence under u.v. light and (*c*) by isolation on ion-exchange columns by procedures essentially similar to those described for *p*-aminobenzoic acid. Fewer experiments were done with *m*-nitrobenzoic acid, and the arylamine produced in cultures grown on this substrate was considered by analogy to be *m*-aminobenzoic acid.

Removal of p-*aminobenzoic acid by washed suspensions*

The disappearance of *p*-aminobenzoic acid was studied by incubating suspensions of adapted and unadapted cells of *N. erythropolis* with *p*-aminobenzoic acid in phosphate buffer, pH 7·0, under sterile conditions with shaking at 30°. Since this experiment extended over 48 hr. sterile precautions were observed when samples were withdrawn at intervals, analysed for *p*-aminobenzoic acid and examined chromatographically for hydroxy compounds. The marked difference in the behaviour of the two types of cells is shown in Fig. 2. Failure of cells grown with *p*-nitrobenzoic acid to metabolize *p*-aminobenzoic acid immediately provides further evidence that this acid is not on the direct pathway of *p*-nitrobenzoic acid degradation, although the ultimate metabolites of both *p*-nitrobenzoic acid and *p*-aminobenzoic acid may be the same. No hydroxy compounds were detected but this may be due to their further metabolism in phosphate buffer (cf. Cartwright & Cain, 1959).

Reduction with cell-free extracts

Cell-free extracts of *N. erythropolis* and *N. opaca* were no longer able to oxidize the *para*- and *ortho*-isomers respectively of nitrobenzoic acid but still brought about reduction of these substrates although their activity was much less than that of the intact cells. Nevertheless, the reduction occurring was sufficient to enable investigations of the process to be made.

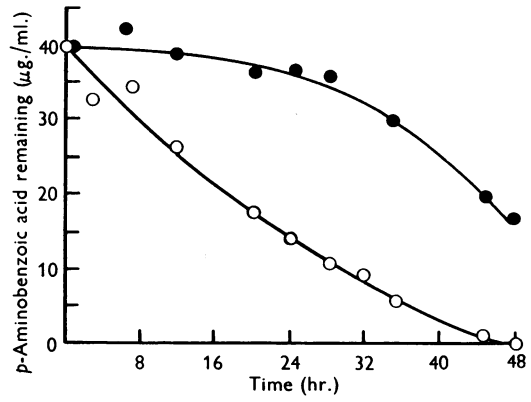

Fig. 2. Disappearance of *p*-aminobenzoic acid on incubation with washed suspensions of *Nocardia erythropolis*. Adapted and unadapted cells were grown as described in Fig. 1 and harvested under aseptic conditions. The organisms were incubated at 30° with the aeration conditions described in Fig. 1 in sterilized incubation mixtures (100 ml. final volume) containing: *p*-aminobenzoic acid, 10 μmoles; 0·067 M-phosphate buffer, pH 7·2; initial dry wt. of adapted cells was 90 mg. and of unadapted cells was 93·2 mg. Portions (3 ml.) were taken with sterile precautions, deproteinized with 10% trichloroacetic acid (0·5 ml.) and estimated for residual *p*-aminobenzoic acid (1 μg./ml. is equivalent to 7·25 μm-moles/ml.). ●, Adapted cells; ○, unadapted cells.

Table 2. *Reduction of* p-*nitro-*, p-*nitroso- and* p-*hydroxylamino-benzoic acid by extracts of* Nocardia erythropolis *CA* 2

Each flask contained: substrate, 5 μmoles; $MgSO_4$, 10 μmoles; DPN, 0·4 μmole; L-cysteine, 4 μmoles; 0·1 M-phosphate buffer, pH 7·0, 2 ml.; extract, 4·4 mg. of protein (Expt. 1), 2·8 mg. of protein (Expt. 2); water to 4 ml. Since *p*-hydroxylaminobenzoic acid gives a colour reaction with the Bratton–Marshall test (Bauer & Rosenthal, 1944), control flasks containing (*a*) substrate + boiled enzyme, or (*b*) no substrate and active enzyme, to correct for extraneous colour and endogenous arylamine respectively were included. After deproteinizing with 1 ml. of 10% (w/v) trichloroacetic acid the contents were analysed for *p*-aminobenzoic acid. The reaction proceeds at constant rate, therefore results are expressed as total arylamine formed/hr./mg. of protein extract. Expt. 1 was performed with extracts from actively growing cells (log phase) and Expt. 2 with extracts from 6-day-old cells (stationary phase).

Substrate	*p*-Aminobenzoic acid produced (μm-moles/hr./mg. of protein) Expt. 1	Expt. 2
p-Nitrobenzoic acid	6·2	3·4
p-Nitrosobenzoic acid	7·0	4·5
p-Hydroxylaminobenzoic acid	6·4	3·1
None	0·2	0·1

Stepwise reduction of the nitro group. *p*-Hydroxylaminobenzoic acid and *p*-nitrosobenzoic acid are likely intermediates between *p*-nitrobenzoic acid and *p*-aminobenzoic acid. Cell-free extracts of *N. erythropolis* grown with *p*-nitrobenzoic acid were prepared by ultrasonic disintegration, under the conditions described by Cartwright & Cain (1959), and incubated with *p*-nitrobenzoic acid and *p*-hydroxylaminobenzoic acid under the conditions given in Table 2. In these experiments the correction of Glazko *et al.* (1949) for extraneous colour obtained in the controls with boiled extract was applied in estimating the content of *p*-aminobenzoic acid. The results agree with reduction proceeding via nitroso and hydroxylamino compounds, the rates of reduction of these compounds being comparable with or higher than that of *p*-nitrobenzoic acid itself.

Production of p-*nitrosobenzoic acid and* p-*hydroxylaminobenzoic acid from* p-*nitrobenzoic acid.* The role of nitroso- and hydroxylamino-benzoic acid as intermediates in the reduction process was demonstrated by the action of extracts of *N. erythropolis* on *p*-nitrobenzoic acid followed by chromatography of the reaction mixtures. *p*-Aminobenzoic acid and hydroxylaminobenzoic acid were visualized by direct spraying with Ehrlich's reagent; nitrosobenzoic and *p*-nitrobenzoic acid were reduced by an acidic 0·5% $SnCl_2$ spray before using Ehrlich's reagent. The nitroso compound, which moved close to *p*-nitrobenzoic acid, was more clearly distinguished by spotting at 5 mm. intervals on a separate sheet with microdroplets of a solution of diphenylamine in conc. H_2SO_4, which produced a bright-red colour with nitrosobenzoic acid but had no effect on the other metabolites. Table 3 shows the progressive reduction of *p*-nitrobenzoic acid recorded chromatographically.

Some properties of the reductase complex. The reductive capacity of cell-free extracts of both *N. opaca* and *N. erythropolis* prepared by ultrasonic disintegration was lower than that of an equivalent quantity of washed cells. However, the extent of reduction in the absence of added H-donors was dependent on the amount of extract present and more evidence for the enzymic nature of the activity was provided by the observation of only very slight arylamine production with a boiled extract. The optimum pH for the reduction of *p*-nitrobenzoic acid was 7·0 in both phosphate and tris buffer, but the rate of reduction as well as the total yield of arylamine was greater in the presence of phosphate. Centrifuging an extract at 104 000 *g* for 20 min. produced a particle fraction with negligible activity after washing, but the supernatant soluble portion remained active. Of several thiols tried, only L-cysteine had a stimulatory effect on the reductase activity, but was not essential (cf. Saz & Slie, 1954*b*). Other reducing agents, including ascorbic acid, did not affect the reaction in any way.

The amounts of arylamine produced by extracts of *N. erythropolis* grown on glucose or *p*-nitrobenzoic acid, corrected for protein concentration, were approximately the same, this finding being explicable on the grounds that reduction was not due to a specific enzyme but was a result of the dehydrogenase activity of other enzymes for which the nitro group acts as H-acceptor (Nason, 1956). Support for this concept was obtained from a study of the effect of extracts on other nitro compounds (Table 4), from which it appears that, whereas

Table 3. *Progressive reduction of* p-*nitrobenzoic acid by* Nocardia *extracts*

To the main compartment of each of four Thunberg tubes, each fitted with a side arm, was added: sodium *p*-nitrobenzoate, 20 μmoles; FAD, 2 μmoles; $MgSO_4$, 10 μmoles; DPNH, 40 μmoles; 0·05 M-tris buffer, pH 7·2, 7 ml. The hollow cap contained cell-free extract, 20·4 mg. of protein; the side arm contained 1 ml. of 10% (w/v) trichloroacetic acid. A control tube contained boiled enzyme. The tubes were evacuated and refilled with O_2-free N_2 three times, the contents of cap and main compartment were mixed and incubation was carried out at 30° with gentle agitation. Tubes were removed at 0, 2, 5 and 10 hr.; the reaction was stopped by tipping in the trichloroacetic acid before opening the tubes, and the protein precipitate removed by centrifuging. The supernatants were extracted with cold ether (3 × 10 ml.) and solvent was removed under reduced pressure; the residue was taken up in 0·5 ml. of ethanol. Portions (0·1 ml.) were applied, together with authentic markers, to Whatman no. 542 paper and the chromatograms run overnight in solvent mixture B. Intensity of spots were estimated visually as −, ±, +, + + etc.

	Compounds present			
Time (hr.)	*p*-Nitrobenzoic acid	*p*-Nitrosobenzoic acid	*p*-Hydroxylamino-benzoic acid	*p*-Aminobenzoic acid
0	+ + + +	−	−	−
2	+ + +	+	±	±
5	+	+ +	+	+ + +
10	±	+	±	+ + + +
10 (boiled enzyme)	+ + + +	±	−	±

highest activity was associated with substrates containing both the carboxyl and nitro group, the activity was markedly non-specific.

Cofactors in nitro reduction. Considerable reduction of nitrobenzoate was achieved in the absence of added H-donors, indicating a high level of endogenous H-donors and the dehydrogenase system(s) making these available. Dialysis of the supernatant fraction from extracts centrifuged at 104 000 *g* for 20 min. against distilled water produced a progressive fall in reductase activity to a level as low as 25 % of the original after 48 hr. at 0–4°. The activity was completely restored by the addition of undialysed boiled extract and to the extent of 70–80 % by DPN in catalytic quantities; TPN was ineffective with extracts of both species. It is apparent therefore that dialysis of this duration has little effect on the endogenous H-donor and that the loss of activity is the result of removal of the carrier, DPN. The effects of the addition of these and other possible cofactors are shown in Table 5. There was a linear relationship between recovery of activity and added DPN with 48 hr. dialysed extracts up to a concentration of 1 mM-DPN.

Table 4. *Non-specific nitroreductase activity of extracts of* Nocardia erythropolis *CA* 4

Each flask contained: substrate (as shown); L-cysteine, 4 μmoles; 0·05 M-tris buffer, pH 7·2, 2 ml.; extract, 4·1 mg. of protein; water to 4 ml. Temp. 30°; incubation time, 10 hr.; aerobic conditions. Arylamine was estimated as *p*-aminobenzoic acid after deproteinization with 10 % (w/v) trichloroacetic acid, 0·5 ml. The organism had been grown upon *p*-nitrobenzoic acid.

Substrate	Amount of substrate (μmoles)	Arylamine formed (μm-moles/ml.)
m-Dinitrobenzene	Saturated solution	50
2:4-Dinitrobenzoic acid	10	91
Nitrobenzene*	10	12
2:4-Dinitrophenol	10	5
2:5-Dinitrophenol	10	10
'Nitrofurazone'	5	4
Chloramphenicol†	3	6
2-Fluoro-4-nitrobenzoic acid	10	62
o-Nitrobenzoic acid‡	10	7
m-Nitrobenzoic acid‡	10	29
p-Nitrobenzoic acid	10	31
3-Hydroxy-4-nitrobenzoic acid	5	34

* Prepared as a stable emulsion by ultrasonic irradiation.
† Pure crystalline compound supplied by Parke Davis and Co. Ltd.
‡ Estimated as the *o*- and *m*-aminobenzoate.

Table 5. *Stimulation of activity by added cofactors*

Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; 0·05 M-tris buffer, pH 7·2, 2 ml.; extract (dialysed for 84 hr.), 4·3 mg. of protein; cofactors to final concentration shown; water to 4 ml. Temp. 30°; incubation time, 17 hr.; aerobic conditions.

Addition	Concn. of addition (μmoles/flask)	*p*-Aminobenzoic acid (μm-moles/ml.)
None	—	21
Boiled undialysed extract	0·4 ml./flask	50
DPN	0·4	42
Adenosine triphosphate	20·0	12
Adenosine diphosphate	20·0	12
Adenosine diphosphate + K_2HPO_4	20·0 40·0	30
TPN	0·4	18
TPN	4·0	21
Undialysed extract used as enzyme (no additions)	—	40

Nocardia extracts dialysed for 80–96 hr. (or more) required the addition of a dibasic acid in addition to DPN for the restoration of full activity. L-Malate, fumarate, succinate and aspartate were the most effective. These acids and DPN apparently act by maintaining the supply of DPNH, since DPNH in substrate quantities under both aerobic and anaerobic conditions replaced the stimulation of nitroreductase by an acid + DPN (Table 6). Malic dehydrogenase, fumarase and succinic dehydrogenase activities have all been observed in these extracts (unpublished results) and aspartase has previously been found in *N. erythropolis* (Cain, 1958*a*). The acids most likely act therefore by conversion into malate, which maintains DPN in the reduced form through malic dehydrogenase activity. Cytochrome *c* (0·1 mM) did not influence the stimulatory effect of succinate on dialysed extracts.

The stimulatory effect of flavins on arylamine production by extracts of *Nocardia* species dialysed against water was first observed with a boiled pig-heart preparation with 48 hr.-dialysed extracts, suggesting that flavins are involved in H-transport. This effect was also examined with FAD and FMN. Fig. 3 shows that only FAD stimulated arylamine production by *N. erythropolis* extracts, which had been dialysed against an acid solution for 72 hr. to effect a more rigorous removal of endogenous flavin. Extracts of *N. opaca* responded to FMN to some extent, but riboflavin was without effect on extracts of both species. It was observed that TPN would not substitute for DPN in flavin-enriched extracts and that flavins had little effect on fresh crude extracts.

Effect of inhibitors. Both hydroxylamine and nitrite acted as inhibitors of nitroreductase activity, the effect of the former being comparable in magnitude with that of *p*-chloromercuribenzoic acid (Table 7). By analogy with their effects upon nitrite and hydroxylamine reductase (Taniguchi, Sato & Egami, 1956), hydroxylamine probably affects the conversion of nitroso- into hydroxylamino-benzoate and nitrite the second stage, i.e. hydroxylamino- to amino-benzoate; chromato-

Table 6. *Requirements of an extensively dialysed extract for a reduced diphosphopyridine nucleotide source*

Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; L-cysteine, 4 μmoles; $MgSO_4$, 10 μmoles; 96 hr.-dialysed extract, 7·4 mg. of protein; 0·1 M-phosphate buffer, pH 7·0, 2 ml. Additions (or water) as shown; total volume 4·5 ml. Temp. 30°; incubation time, 14 hr.; aerobic conditions.

Additions	Concn. of addition (μmole/ml.)	DPN added (μmole/ml.)	Arylamine formed (μm-moles/ml.)
None	—	—	18
None	—	0·1	29
Boiled undialysed extract	0·4 ml./flask	—	34
L-Malate	1	0·1	37
DL-Aspartate	1	0·1	33
Fumarate	1	0·1	43
Succinate	1	0·1	38
L-Malate + DPNH	1 + 1	0·1	39
DPNH	1	—	38
DPNH (boiled enzyme)	1	—	7

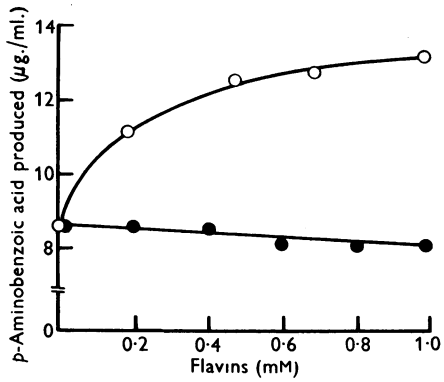

Fig. 3. Stimulation of *Nocardia erythropolis* nitroreductase by flavins. Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; DPN, 4 μmoles; L-cysteine, 4 μmoles; extract (dialysed for 72 hr. against frequent changes of water made to pH 5·5 with N-acetic acid), 5·25 mg. of protein; flavins were added as shown; water to 4 ml. The reaction mixtures were made up in diffuse light, the bath was covered with black cloth and the incubation performed overnight in the dark. Incubation time, 12 hr.; temp. 30°. *p*-Aminobenzoic acid: 1 μg./ml. is equivalent to 7·25 μm-moles/ml. ○, FAD; ●, FMN.

graphy of incubation mixtures inhibited with either hydroxylamine or nitrite showed the presence of *p*-nitrosobenzoic acid. The effect of hydroxylamine shown in the Table is quite different from that demonstrated in oxidative metabolism of *p*-nitrobenzoic acid (Cartwright & Cain, 1959), where nitrite was probably split off and concentrations of hydroxylamine as low as 0·1 mM were completely inhibitory.

Metal requirement in nitro group reduction. Separate samples of freshly prepared extracts of *N. erythropolis* were dialysed against KCN, 1:10-phenanthroline and ethylenediaminetetra-acetic acid (EDTA) (all mM) for 48 hr. at 4°. There was a marked fall in activity with these agents, and almost

Table 7. *Inhibition of reduction of* p-*nitrobenzoate*

Each Warburg flask contained: *p*-nitrobenzoic acid, 5 μmoles; extract, 4·3 mg. of protein; 0·05 M-tris buffer, pH 7·2, 2·5 ml.; inhibitor to concentration shown; L-cysteine, 4·5 μmoles; water to 4·5 ml. Temp. 30°; incubation time, 17 hr. Inhibition is expressed as the percentage decrease in arylamine of that produced in absence of inhibitor.

Addition	Concn. of addition (mM)	Inhibition (%)
Hydroxylamine	10	86·3
	5	95·3
	0·1	50·0
	0·01	2·0
Sodium nitrite	5	32·5
	1	16·0
	0·1	1·5
	0·01	0
p-Chloromercuribenzoic acid	1	80·0
	0·01	7·5

complete inhibition with phenanthroline; KCN was the least effective, causing 35–40% inhibition. Table 8 shows the reversal of this inhibition by addition of metal ions to extracts prepared from actively growing and stationary-phase cells. Mg^{2+}, Mn^{2+} and Fe^{2+} ions are particularly effective out of several dibasic ions tested, a result which has been repeatedly confirmed for different cell batches over a period of 2 years.

Growth experiments in media rendered metal-free by the methods of Waring & Werkman (1943) showed that, with *N. erythropolis*, Mn^{2+} ion, involved in the *Escherichia coli* nitroreductase (Saz & Slie, 1954*a*), was without effect on either growth or arylamine production in the presence of excess of Fe^{2+} ions (1 μg./ml.), but increase in Fe^{2+} ions from zero to 0·02 μg./ml., in the presence of 0·1 μg. of Mn^{2+}/ml., increased the arylamine production in cultures 40-fold but growth was increased only 13-fold, whereas increase in Fe^{2+} from zero to

Table 8. *Reversal of the effects of dialysis against chelating agents by added metal ions*

Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; L-cysteine, 4 μmoles; DPN, 0·4 μmole; added metal ion, 4 μmoles; 0·1 M-phosphate buffer, pH 7·0, 1·5 ml.; dialysed extract, 3·3 mg. of protein (Expt. 1) and 2·8 mg. of protein (Expt. 2). Temp. 30°; incubation time, 18 hr.; aerobic conditions.

All metals were added as sulphates except $FeCl_6,6H_2O$ and Na_2MoO_4.

Expt. 1 was performed with extracts from 6-day-old (stationary-phase) cells and Expt. 2 with 2-day-old (log-phase) cells. The figures given represent the stimulation, which is expressed as the arylamine produced compared with control in the absence of metal (= 100).

	Chelating agent used in dialysis							
	KCN		1:10-Phenanthroline		EDTA		8-Hydroxyquinoline	
Added metal ion	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Fe^{2+}	—	100	325	357	182	129	106	—
Fe^{3+}	—	—	118	100	100	—	197*	—
Mg^{2+}	—	177	118	100	330	290	—	—
Mn^{2+}	—	100	120	171	180	224	110	—
MoO_4^{2-}	—	100	110	100	163	100	121	—

* There was significant reduction to Fe^{2+} ions in the presence of cysteine over the duration of the experiment.

0·04 μg./ml. increased arylamine 80-fold but growth only 50-fold.

Attempts to separate components of the enzyme complex. All efforts to separate the distinct enzymes responsible for the stages nitro to nitroso, nitroso to hydroxylamino and hydroxylamino to amino by standard techniques of protein purification failed completely. Only an overall concentration of the total reductase activity was achieved.

DISCUSSION

The experiments described establish the ability of growing cultures, washed cell suspensions and cell-free extracts of *Nocardia* species, and of washed cell suspensions of *P. fluorescens* NC 3, to reduce the nitro group of the nitrobenzoic acids, in particular of the *para*-isomer, to the amino group with the accumulation of the corresponding aminobenzoic acids, which, after reaching a maximum concentration, slowly disappear. The ultimate fate of the amino acids has not been fully established in the present study. A consideration of our findings, together with the evidence of oxidative metabolism (Cartwright & Cain, 1959), indicates that reductive dissimilation is a subsidiary process. *p*-Aminobenzoic acid does not lie on the direct oxidative pathway because no appreciable oxidation of this substrate has been observed with cells grown with *p*-nitrobenzoic acid; it is removed far more rapidly by cells grown with glucose than by cells grown with *p*-nitrobenzoic acid. In addition, *N. erythropolis* grows poorly on *p*-aminobenzoic acid as sole carbon, nitrogen and energy source compared with its growth on *p*-nitrobenzoic acid. This further confirms the difference between *p*-nitrobenzoate metabolism in *Nocardia* (Cartwright & Cain, 1959) and in *Pseudomonas*, in which Durham (1958) indicated that *p*-aminobenzoic acid was a direct intermediate.

Our previous findings suggest that direct oxidation of *p*-nitrobenzoic acid yields nitrite and *p*-hydroxybenzoic acid, which is oxidized further; reduction of the nitro group which occurs slowly at the same time results in the gradual accumulation of *p*-aminobenzoic acid, which eventually reaches a level sufficient to induce some *p*-aminobenzoic acid-metabolizing activity in the cells. The existence of a prolonged induction period before cells grown with *p*-nitrobenzoic acid will rapidly remove *p*-aminobenzoic acid, whereas cells grown on glucose do so immediately, could be explained by the existence of a common precursor of the *p*-nitrobenzoic acid and *p*-aminobenzoic acid-metabolizing enzymes which is almost completely removed during growth on *p*-nitrobenzoic acid. In any case, if *p*-nitrobenzoic acid metabolism proceeded directly through *p*-aminobenzoic acid, the release of the nitro group as nitrite would be ruled out. Evidence in support of the reduction of the nitro group not being a primary reaction is provided by the observations that *Nocardia* M 1, which also produces arylamine, releases 70% of nitro nitrogen as nitrite, and that cultures of *N. opaca* and *N. erythropolis* grown, respectively, on *o*- and *p*-nitrobenzoic acid also yield nitrite in small amounts. The difference between the nitrite levels in *Nocardia* M 1 and the two other species was explained by Cartwright & Cain (1959).

The reduction of the nitro group by *Nocardia* species probably proceeds via the corresponding nitroso and hydroxylamino compounds. The facts that both are reduced by extracts at similar rates to *p*-nitrobenzoic acid and that their presence has been demonstrated chromatographically as products of the action of extracts on *p*-nitrobenzoic acid itself is regarded as evidence of their role. Saz & Martinez (1956) have found that *p*-nitrosobenzoic acid appears in the reduction of *p*-nitrobenzoic acid

by a strain of *E. coli*, and Yamashina, Shikata & Egami (1954) presented chromatographic evidence for the formation of the corresponding *o*-nitroso- and hydroxylamino-benzoic acid from *o*-nitrobenzoic acid by extracts of *Bacillus pumilus*. This stepwise reduction of the nitro group to amino group is obviously of widespread occurrence, hydroxylamino compounds having also been demonstrated and isolated from animal tissues (Beuding & Jolliffe, 1946) and *Neurospora crassa* (Nason, 1956).

Although Hondo, Otsuka & Shimoda (1956) reported an apparently specific nitroreductase, most nitroreductase activity appears to be non-specific, being the result of known enzyme systems oxidizing their substrates and using the nitro group as an H-acceptor, e.g. succinic dehydrogenase (Westfall, 1943), alcohol and formic dehydrogenases (Greville & Stern, 1935) and yeast diaphorase (Brodie & Gots, 1952). The ability of extracts of any one of several *Nocardia* species to reduce a variety of nitro compounds, nitroso- and hydroxylamino-benzoic acid, and the additional property of an extract from an organism grown on one isomer of nitrobenzoic acid to reduce the other two (Cain, 1958*a*, and unpublished results), together with the failure to separate enzyme fractions capable of mediating in only one reaction of the chain, suggests that the *Nocardia* enzymes are likewise non-specific.

The reductase activity of these organisms has other properties in common with the enzymes previously found in moulds and bacteria. The nitroreductase of *E. coli* was DPN-mediated (Saz & Slie, 1954*b*), and like that of *N. erythropolis* had a flavin requirement (Saz & Martinez, 1956). An FAD-linked *m*-dinitrobenzene reductase in *Neurospora crassa* has been reported (Zucker & Nason, 1955). Several nitrite reductases responsible for reducing nitrogen at the same oxidation level as in nitro compounds are also flavin-linked; those from *Neurospora* (Nicholas & Nason, 1954) and *E. coli* (Nicholas & Nason, 1955) have both flavin and molybdenum requirements. This is worthy of note in view of the apparent identity of the chloramphenicol nitroreductase and nitrite reductase of *Streptococcus haemolyticus* (Egami *et al.* 1951) and the inhibition of *N. erythropolis* nitroreductase by hydroxylamine, which characteristically inhibits nitrite reductase (Taniguchi *et al.* 1956).

Yamashina (1954), Yamashina *et al.* (1954) and Villanueva (1959) have all noted the inhibitory effect of potassium cyanide and sodium azide on nitroreductases in bacteria; our results agree with this. Villanueva found that EDTA, 8-hydroxyquinoline and $\alpha\alpha'$-dipyridyl had but little effect on a nitroreductase from a *Nocardia* species but the first two compounds and especially 1:10-phenanthroline caused extensive inhibition in the nitroreductase from *N. erythropolis*. It is not clear from Villanueva's preliminary report whether the chelating agents were added to the reaction mixture or whether, as in our experiments, the extracts were dialysed against such agents. We have found that the latter method is the more rigorous. The differences may thus be merely qualitative. The addition of metal ions to crude extracts had no effect, but addition to extracts dialysed against chelating agents revealed the stimulation of nitroreductase given by Mg^{2+}, Mn^{2+} or Fe^{2+} ions. Fe^{2+} was the only metal effective in increasing arylamine in growth experiments.

The variations in metal-stimulation responses given by extracts dialysed against the different reagents is probably a result of the metal–chelate association constants. 1:10-Phenanthroline, for instance, is particularly effective for Fe^{2+} ions. Addition of Fe^{2+} ions to a phenanthroline-dialysed extract therefore results in a greater stimulation than would result from addition of other bivalent ions.

Other minor pathways of nitrobenzoate metabolism by reduction of the nitro group exist in these organisms. 3-Hydroxy-4-aminobenzoate is produced by *N. erythropolis* from *p*-nitrobenzoic acid, and *N. opaca* gives 3-hydroxyanthranilic acid from *o*-nitrobenzoic acid (Cain, 1958*b*). These compounds are also produced by the action of dried preparations of the organisms on the corresponding hydroxynitrobenzoic acids. The order of hydroxylation and reduction has not been deduced but the work of Partridge, Bonner & Yanofsky (1952) suggests that in micro-organisms there is no direct hydroxylation of anthranilic acid as observed in mammalian metabolism by Kotake & Shirai (1953).

SUMMARY

1. Growing cultures, washed cell suspensions and cell-free extracts of *Nocardia* species and a strain of *Pseudomonas fluorescens* reduce the nitrobenzoic acids to the corresponding aminobenzoic acids.

2. Reductive dissimilation is a subsidiary process, not a primary mode of attack on the nitro group, and the aminobenzoic acids do not lie on the direct oxidative pathway of nitrobenzoic acid metabolism.

3. Reduction of the nitro group to the amino level probably proceeds in *Nocardia erythropolis* via the corresponding nitroso- and hydroxylamino-benzoic acid.

4. No separation of the enzymes responsible for the individual steps in the reductive process was obtained with standard protein-fractionation techniques.

5. The nitroreductase is non-specific and requires reduced diphosphopyridine nucleotide as H-donor. Its activity is stimulated by flavinadenine dinucleotide and inhibited by hydroxylamine and nitrite.

6. Dialysis against ethylenediaminetetra-acetic acid, 1:10-phenanthroline, 8-hydroxyquinoline and potassium cyanide causes extensive inhibition and reveals the requirement for a bivalent metal. Mn^{2+}, Mg^{2+} and, in particular, Fe^{2+} ions are active. The effect of the last-named was also observed in experiments with metal-free media.

7. The significance in the overall metabolism of several hydroxynitrobenzoic acids isolated on chromatograms from experiments with *Nocardia* species could not be deduced.

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Studies on the Succinate–Neotetrazolium Reductase System

ACTIVATION BY VITAMIN K_3

BY T. F. SLATER*
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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The formation of insoluble formazans by the enzymic reduction of tetrazolium salts has been extensively used in the histological demonstration of dehydrogenase activity. Recently several reports have been concerned with the application of this procedure to the quantitative estimation of dehydrogenase activity in tissue homogenates (Kun & Abood, 1949; Sprinz & Waldschmidt-Leitz, 1953; Zöllner & Rothemund, 1954; Shelton & Rice, 1957; Sourkes & Lagnado, 1957).

The mechanism of the reaction is still unknown, as is the point(s) at which tetrazolium couples with

* Beit Memorial Fellow.