The Identification and Biosynthesis of Siderochromes Formed by *Micrococcus denitrificans*

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1. *Micrococcus denitrificans* excretes three catechol-containing compounds, which can bind iron, when grown aerobically and anaerobically in media deficient in iron, and anaerobically in medium with a high concentration of Ca^{2+} . 2. One of these compounds was identified as 2,3-dihydroxybenzoic acid (compound I), and the other two were tentatively identified as N^1N^8 -bis-(2,3-dihydroxybenzoyl)spermidine (compound II) and 2-hydroxybenzoyl-*N*-L-threonyl- N^4 [N^1N^8 -bis-(2,3-dihydroxybenzoyl)]spermidine (compound III). 3. The equimolar ferric complex of compound III was prepared; compound III also forms complexes with Al^{3+} , Cr^{3+} and Co^{2+} ions. 4. Cell-free extracts from iron-deficient organisms catalyse the formation of compound II from 2,3-dihydroxybenzoic acid and spermidine, and of compound III from compound II, L-threonine and 2-hydroxybenzoic acid; both reactions require ATP and dithiothreitol, and Mg^{2+} stimulates activity. The enzyme system catalysing the formation of compound II has optimum activity at pH 8.8. Fe^{2+} (35 μM), Fe^{3+} (35 μM) and Al^{3+} (65 μM) inhibit the reaction by 50%. The enzyme system forming compound III has optimum activity at pH 8.6. Fe^{2+} (110 μM), Fe^{3+} (110 μM) and Al^{3+} (135 μM) inhibit the reaction by 50%. 5. At least two proteins are required for the formation of compound II, and another two proteins for its conversion into compound III. 6. The changes in the activities of these two systems were followed after cultures became deficient in iron. 7. Ferrous 1,10-phenanthroline is formed when a cell-free extract from iron-deficient cells is incubated with the ferric complex of compound III, succinate, NADH and 1,10-phenanthroline under N_2 .

Although iron is present in abundance in the earth's crust, it is not readily available to living organisms because the Fe^{3+} ion is very insoluble at neutral pH values. To overcome this problem many micro-organisms excrete ligands which complex with Fe^{3+} . The soluble ferric complex is then taken up by the micro-organism, the iron is released enzymically, and incorporated into porphyrins and proteins. A large number of microbial iron-transport compounds, which have been named siderochromes, are known (for review see Neilands, 1972). Their formation by micro-organisms is repressed when iron is present in the culture media, but large amounts accumulate when the media are deficient in iron. Two main types of siderochromes have been described, those containing hydroxamic acid residues, and those containing catechol residues. Relatively few compounds of the latter type, which appear to be formed only by prokaryotic micro-organisms, are known (Neilands, 1972). Cyclotris-(2,3-dihydroxybenzoyl-*N*-L-seryl) (enterobactin, enterochelin) is produced by *Aerobacter aerogenes* and *Escherichia coli* (O'Brien & Gibson, 1970) and by *Salmonella typhimurium* (Pollack & Neilands, 1970), 2,3-dihydroxybenzoylglycine (itoic acid) by *Bacillus subtilis* (Ito & Neilands,

1958), 2,3-dihydroxybenzoylthreonine by *Klebsiella oxytoca* (Korth, 1970) and N^2N^6 -bis-(2,3-dihydroxybenzoyl)-L-lysine by *Azotobacter vinelandii* (Corbin & Bulen, 1969). A number of these organisms also excrete 2,3-dihydroxybenzoic acid (Ratledge & Chaudry, 1971). Bryce & Brot (1972) have separated and partially purified three enzymes required for the synthesis of enterochelin from 2,3-dihydroxybenzoic acid and L-serine.

When *Micrococcus denitrificans* was grown aerobically and anaerobically in iron-deficient media catechols accumulated in the media. Three compounds were isolated and identified as 2,3-dihydroxybenzoic acid (compound I), N^1N^8 -bis-(2,3-dihydroxybenzoyl)spermidine (compound II) and 2-hydroxybenzoyl-*N*-L-threonyl- N^4 -[N^1N^8 -bis-(2,3-dihydroxybenzoyl)]spermidine (compound III). Cell-free extracts from iron-deficient *M. denitrificans* were shown to catalyse the formation of compound II and compound III. The properties of these enzyme systems were investigated. Cell-free extracts of iron-deficient cells were also found to catalyse the reduction of iron in the ferric complex of compound III to the ferrous state. A preliminary account of some of these results has been published (Tait, 1974).

Experimental

Chemicals

ATP, CoA, NAD⁺, NADH and NADPH were obtained from C. F. Boehringer und Soehne GmbH, Mannheim, Germany; 2-mercaptoethanol, dithiothreitol and D-threonine from Sigma (London) Chemical Co. Ltd., London S.W.6., U.K.; DEAE-cellulose and CM-cellulose from Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, U.K.; Zeo-Karb 225 from The Permutit Co. Ltd., Isleworth, Middx., U.K.; silicic acid (100 mesh) from Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.; putrescine dihydrochloride (1,4-diaminobutane dihydrochloride) and cadaverine dihydrochloride (1,5-diaminopentane dihydrochloride) from Roche Products Ltd., Welwyn Garden City, U.K.; N-(3-aminopropyl)-1,3-diaminopropane, spermidine [N-(3-aminopropyl)-1,4-diaminobutane], spermine and 2,3-dihydroxybenzoic acid were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. 2,3-Dihydroxybenzoic acid was recrystallized from hot water before use. L-[U-¹⁴C]Threonine and ¹⁴C-labelled spermidine hydrochloride were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were commercial preparations of the purest grade available.

Methods

Growth of organisms. *Micrococcus denitrificans* (N.C.I.B. 8944) was obtained from Professor J. G. Morris, Department of Botany and Microbiology, University College of Wales, Aberystwyth. Organisms were maintained on slopes containing (per litre): bacteriological peptone, 4g; yeast extract, 2g; KH₂PO₄, 10g; glucose, 10g (sterilized separately); KNO₃, 20g; agar, 20g; adjusted to pH 6.8 with H₂SO₄ (Scholes & Smith, 1968). Boiling tubes containing 40ml of this complex medium without agar were inoculated from slopes and grown for 24h at 30°C to provide inocula for the large-scale growth of organisms; 4ml was used to inoculate each litre of medium.

Organisms were grown anaerobically in defined nitrate medium and aerobically in defined nitrate or ammonia medium as described by Tait (1973). All components of the media were analytical reagent-grade chemicals. The media contained (per litre): succinic acid, 5.9g; NaOH, 4.0g; KH₂PO₄, 4.0g; Na₂HPO₄, 4.9g; MgSO₄·7H₂O, 0.2g; Na₂MoO₄·2H₂O, 0.15g; KNO₃, 10.1g, or NH₄Cl, 1.6g. After autoclaving the media sterile solutions of ferric citrate (Chang & Morris, 1962), CaCl₂, CuSO₄ and MnSO₄ were added as required. Ferric citrate was added to give a final concentration of 20 μM. For growth under conditions of iron deficiency ferric citrate was added to give a

final concentration of 0.8 μM. For anaerobic growth in nitrate medium MnSO₄, CaCl₂ and CuSO₄ were added to give concentrations of 4.5 μM, 9 μM and 0.1 μM respectively. In some experiments organisms were grown anaerobically in a high-calcium medium which contained 180 μM-CaCl₂. For aerobic growth in nitrate medium and in ammonia medium MnSO₄ was added to a concentration of 4.5 μM.

Organisms were grown at 30°C for up to 50h. For anaerobic growth 0.5–5-litre conical flasks filled almost to the top with nitrate medium were used; N₂ is evolved during growth. For aerobic growth air was bubbled through 1 litre of medium contained in a 2-litre conical flask, or 300ml conical flasks containing 100ml or 150ml of medium were shaken continuously at 140 strokes per min.

After growth, organisms were harvested by centrifugation for 10min at 15000g at 4°C and resuspended in cold 0.05M-Tris-HCl buffer, pH 8.8 (0.05M with respect to Tris), to a volume approximately one-tenth of that of the original culture. Suspensions were frozen and kept at –20°C until required. The culture supernatants were adjusted to pH 2 with HCl and freeze-dried. The dry residue was kept at –20°C until required.

Preparation of enzyme extracts. Suspensions of organisms were disrupted in 2–5ml lots by irradiation in an MSE 50W ultrasonic disintegrator operated at maximum output for 3min. Suspensions were kept cool by immersing the tube in crushed ice. The suspensions were then centrifuged at 25000g for 25min and the clear supernatants, termed enzyme extract, were removed. Enzyme extract was either frozen immediately, or after dialysis against 0.05M-Tris-HCl buffer (pH 7.7)–1mM-dithiothreitol at 4°C overnight and stored at –20°C until required.

Determinations. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard. Fractions eluted from columns were monitored for protein by measuring the *E*₂₈₀. Growth of cultures of *M. denitrificans* was determined by measuring protein content, by the method of Lowry *et al.* (1951), of cells which had been harvested and resuspended in 0.05M-Tris-HCl buffer, pH 8.8. Catechols were measured by reaction with Arnou's nitrite-molybdate reagent as described by Corbin & Bulen (1969) by using 2,3-dihydroxybenzoic acid as standard. Amino acids and compounds containing primary amine groups were determined by reaction with ninhydrin as described by Rosen (1957). The presence of primary and secondary amine groups in compounds, and the number of each type present, was determined by reaction with 1-fluoro-2,4-dinitrobenzene as described by Dubin (1960). Spectra of solutions were measured and the *E*₃₅₀/*E*₃₉₀ ratio was calculated. In this method compounds with secondary amine groups give an

E_{350}/E_{390} ratio different from that given by compounds with primary amine groups (Dubin, 1960).

Ascending paper chromatography was done on Whatman no. 1 paper. The solvents used to separate hydroxybenzoic acids were butan-1-ol-pyridine-water (14:3:3, by vol.), benzene-acetic acid-water (125:72:3, by vol.), and 5% (w/v) ammonium formate in 0.5% (v/v) formic acid. Spots were located by examining the chromatogram under u.v. light, and by spraying with 1% (w/v) FeCl_3 in water or with nitroaniline reagent (Smith *et al.*, 1969). The solvents used to separate amino acids and amines were butan-1-ol-acetic acid-water (12:3:5, by vol.), phenol-water (4:1, w/v) and methyl cellulose-propionic acid-water (14:3:3, by vol.), saturated with NaCl (Herbst *et al.*, 1958). Spots were detected by spraying the paper with 0.2% (w/v) ninhydrin in acetone and heating it at 100°C.

High-voltage paper electrophoresis was performed essentially as described by Atfield & Morris (1961). Electrophoresis was done on Whatman 3MM or no. 1 papers (22cm × 56cm) in 1.2% (v/v) formic acid-4.35% (v/v) acetic acid, pH 2.0, or 0.4% (v/v) pyridine-0.19% (v/v) acetic acid, pH 5.2 at 3 kV and 65 mA for 15–30 min. Electrophoresis was also done on Whatman no. 1 papers (25cm × 15cm) in 0.05M-sulphosalicylic acid adjusted to pH 4.0 with NaOH, and in 0.03M-citric acid adjusted to pH 6.5 with NaOH at 150V and 6mA for 3–4 h (Herbst *et al.*, 1958).

The polyamine released by acid hydrolysis of compounds II and III (see below) and spermidine were dansylated as described by Creveling & Daly (1971) and chromatographed on thin layers of silica gel (E. Merck AG, Darmstadt, West Germany) in ethyl acetate-cyclohexane (1:2, v/v) and benzene-triethylamine (5:1, v/v) (Dion & Cohen, 1972).

Radioactivity. Samples were plated on 6.25cm² aluminium planchets, dried and counted at infinite thinness in a Nuclear-Chicago gas-flow counter fitted with a Micromil end-window and operated at the centre of the plateau. Under these conditions 1 μCi of ¹⁴C gave about 500000 c.p.m. Radioactive spots on paper after chromatography or electrophoresis were located by radioautography by using Kodirex X-ray film (Kodak Ltd., London W.C.2, U.K.). Elementary analysis for the carbon, hydrogen and nitrogen content of compounds was done by Mrs. Doris Butterworth, Division of Chemical Standards, National Physical Laboratory, Teddington, Middlesex, U.K. Iron was determined spectrophotometrically, after digestion of the organic iron complex with concentrated H_2SO_4 at 180°C, as described by Ballentine & Burford (1957).

Acid hydrolysis was done in 6M-HCl in evacuated sealed ampoules at 110°C for 24–72 h.

Visible and u.v. spectra were measured with a Unicam SP. 700 spectrophotometer. I.r. spectra were

done on KCl disks, made by pressing a mixture of 500 μg of the compound and 100mg of KCl, in a Unicam SP. 200 spectrophotometer. Optical rotations of solutions were measured at 365, 436, 546, 578 and 589 nm in a Perkin-Elmer 141 Polarimeter.

Enzyme assays. (a) Aminolaevulinic synthase (EC 2.3.1.37) was assayed in undialysed enzyme extracts of *M. denitrificans* exactly as described by Tait (1973). (b) L-Threonine dehydrogenase (EC 1.1.1.103). *Rhodopseudomonas spheroides* (N.C.I.B. 8253) was grown semi-anaerobically in the light as described by Neuberger & Tait (1962). A suspension of cells was disrupted by ultrasonication and centrifuged at 105000g for 2 h. The clear supernatant, containing 8 mg of protein/ml, was removed and used as the enzyme extract. Different amounts of L-threonine (0.5–2 μmol) were incubated with 0.5 μmol of NAD^+ , 75 μmol of Tris-HCl buffer, pH 8.8, and enzyme extract (2.0 mg of protein) in a total volume of 0.75 ml for 1 h at 37°C. The amounts of aminoacetone formed were measured as described by Neuberger & Tait (1962).

Enzymic formation of N^1N^8 -bis-(2,3-dihydroxybenzoyl)spermidine (compound II). The assay mixture contained in a total volume of 0.2 ml: 20 μmol of Tris-HCl buffer, pH 8.8; 1.5 μmol of MgCl_2 ; 2 μmol of dithiothreitol; 1 μmol of ATP; 200 nmol of ¹⁴C-labelled spermidine (124 c.p.m./nmol at infinite thinness); 200 nmol of 2,3-dihydroxybenzoic acid; dialysed enzyme extract (0.5–1.0 mg of protein) from *M. denitrificans*. The mixture was incubated at 37°C for 1 h and then 0.1 ml of 0.5M-HCl and 2.0 ml of ethyl acetate were added. The tubes were mixed thoroughly and then centrifuged. A portion (0.5 ml) of the upper layer was plated and its radioactivity measured.

Enzymic formation of 2-hydroxybenzoyl-N-L-threonyl- N^4 -[N^1N^8 -bis-(2,3-dihydroxybenzoyl)]-spermidine (compound III). (a) The assay mixture contained in a total volume of 0.2 ml: 20 μmol of Tris-HCl buffer, pH 8.8; 1.5 μmol of MgCl_2 ; 2 μmol of dithiothreitol; 1 μmol of ATP; 100 nmol of L-[¹⁴C]-threonine (150 c.p.m./nmol at infinite thinness); 100 nmol of 2-hydroxybenzoic acid; 40 nmol of N^1N^8 -bis-(2,3-dihydroxybenzoyl)spermidine (compound II); enzyme extract (0.5–1.0 mg of protein). After incubation at 37°C for 1 h, 0.1 ml of 0.5M-HCl and 1.0 ml of ethyl acetate were added. The tubes were mixed thoroughly and then centrifuged. A sample (0.5 ml) of the upper layer was plated and its radioactivity was measured. (b) The assay mixture contained in a total volume of 0.2 ml: 20 μmol of Tris-HCl buffer, pH 8.8; 1.5 μmol of MgCl_2 ; 2 μmol of dithiothreitol; 1 μmol of ATP; 100 nmol of L-[¹⁴C]-threonine (292 c.p.m./nmol at infinite thinness); 200 nmol each of spermidine, 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid; enzyme extract (0.5–1.0 mg of protein). After incubation at 37°C for

1 h, 0.1 ml of 0.5M-HCl and 2.0 ml of ethyl acetate were added. The tubes were mixed thoroughly and then centrifuged. A sample (0.5 ml) of the upper layer was plated and its radioactivity was measured.

Results

Formation of catechol-containing compounds by M. denitrificans

When *M. denitrificans* was grown anaerobically in nitrate medium containing 20 μ M-ferric citrate and a high concentration of Ca^{2+} (180 μ M) it was noted that the medium became yellow after about 20 h of growth; with 40 μ M-ferric citrate the medium remained colourless throughout growth. Media also became yellow when cultures were grown anaerobically (with 9 μ M- Ca^{2+}) and aerobically with 0.8 μ M-ferric citrate, but not with 20 μ M-ferric citrate. Yellow media, but not colourless media, turned red on addition of ferric citrate, and it was concluded that under conditions of iron deficiency the cells were forming and excreting one or more compounds capable of complexing with Fe^{3+} . In these yellow media the presence of catechol-containing compounds was detected. It is known that a number of other bacteria when grown in iron-deficient media excrete 2,3-dihydroxybenzoic acid and complexes containing it (Neilands, 1972), whose

phenolic hydroxyl groups chelate Fe^{3+} . The amounts of catechol-containing compounds (Fig. 1) excreted by *M. denitrificans* grown anaerobically in nitrate medium were low compared with those excreted by cells grown aerobically in iron-deficient nitrate or ammonia media. During aerobic growth more was formed when 100 ml of culture was present in a 300 ml conical flask than when 150 ml was present.

Separation of catechol-containing compounds

Preliminary chromatography of culture supernatants showed that there were three catechol-containing compounds. These were separated and purified in the following way. The separation and purification was followed by testing fractions for their content of catechol with Arnow's nitrite-molybdate reagent. Culture supernatant (4 litres; 1500 μ mol of catechol), from a culture grown anaerobically for 48 h in ammonia medium containing 0.8 μ M-ferric citrate, was adjusted to pH 2 with HCl and freeze-dried. The solid residue was extracted four times with 60 ml portions of ethanol; little or no catechols remained in the residue. The pooled ethanol extracts were dried under reduced pressure at 30–40°C. The residue was dissolved in 100 ml of water and the solution (pH 2) was extracted four times with 100 ml portions of ethyl acetate. The aqueous solution, which still contained catechol, was kept for purification of compound II. The pooled ethyl acetate extracts were extracted four times with 50 ml portions of 10% (w/v) NaHCO_3 . The pooled NaHCO_3 extracts were kept for purification of compound I and the ethyl acetate solution was kept for the purification of compound III. Alternatively the culture supernatant was adjusted to pH 2 with HCl, compounds I and III were extracted with ethyl acetate and the residue, containing only compound II, was freeze-dried.

Purification of compound I. The pooled NaHCO_3 extracts were adjusted to pH 2 with HCl, and the catechol was extracted into ethyl acetate. The pooled ethyl acetate extracts were dried over Na_2SO_4 and dried under reduced pressure. The brown residue was dissolved in 10 ml of benzene-ethyl acetate (4:1, v/v) and applied to a column packed with 5 g of silicic acid equilibrated with the same solvent. The column was eluted with benzene-ethyl acetate (4:1, v/v), fractions (10 ml) were collected and those containing catechol were pooled and dried. If the residue was still a brown colour, the chromatography was repeated. The residue was dissolved in the minimum volume of hot water, and on cooling in ice white crystals were formed. These were filtered off, washed with 1 ml of ice-cold water and dried. The yield was 32 mg.

Purification of compound II. The aqueous solution (see above) was freeze-dried. The residue was dissolved in 100 ml of water and mixed with 100 ml of a 50%

Fig. 1. Excretion of catechols by *M. denitrificans*

Organisms were grown anaerobically in nitrate medium containing 9 μ M- Ca^{2+} and 0.8 μ M-ferric citrate (●), or 180 μ M- Ca^{2+} and 20 μ M-ferric citrate (○), or aerobically with 0.8 μ M-ferric citrate in nitrate medium (150 ml/flask) (△), or ammonia medium, 150 ml/flask (■) or 100 ml/flask (□). Samples were taken at the times shown, the cells were removed by centrifugation, and the amounts of catechol in the supernatants were measured.

Fig. 2. Structures of catechols excreted by *M. denitrificans*

Compound I, 2,3-dihydroxybenzoic acid; compound II, *N*¹*N*⁸-bis-(2,3-dihydroxybenzoyl)spermidine; compound III, 2-hydroxybenzoyl-*N*-L-threonyl-*N*⁴-[*N*¹*N*⁸-bis-(2,3-dihydroxybenzoyl)]spermidine.

(v/v) suspension of Zeo-Karb 225 (H⁺) in water. The suspension was filtered, and the resin, which now contained all the catechol, was washed with water. The catechol was eluted from the resin with three 50ml portions of 2M-NH₃ and the pooled extracts were freeze-dried. The residue was redissolved in 5ml of ethanol, and 45ml of water was added. The solution was applied to a column (9cm × 2cm) of CM-cellulose, which had previously been washed with 1M-HCl and then with water. The column was washed with water, and the catechol was eluted with 0.1M-acetic acid. Fractions (10ml) containing catechol were pooled and freeze-dried to give a pale-orange powder. If the powder was markedly orange or brown, which happened on occasions, it was rechromatographed on CM-cellulose in the same way. The yield was 42 mg.

Purification of compound III. The ethyl acetate solution was dried over Na₂SO₄ and dried under reduced pressure. The residue was dissolved in 10ml of benzene-ethyl acetate (4:1, v/v) and applied to a column (1cm diam.) containing 2.5g of silicic acid equilibrated with the same solvent. The column was eluted with benzene-ethyl acetate (4:1, v/v), fractions (10ml) were collected, and those that contained catechol and were colourless were pooled and dried. The residue was dissolved in about 0.5ml of ethanol and gave a white suspension when added with

stirring to 15ml of water. The suspension was freeze-dried to give 77mg of a white powder.

Identification of catechol-containing compounds

Compound I. On the basis of the evidence presented below, compound I is 2,3-dihydroxybenzoic acid (Fig. 2). Elementary analysis: found: C, 54.8; H, 4.0; C₇H₆O₄ requires C, 54.6; H, 3.9%. The melting points of compound I and authentic 2,3-dihydroxybenzoic acid were 208° and 210°C respectively. Compound I and 2,3-dihydroxybenzoic acid had the following properties in common. Their i.r. spectra were identical as were their u.v. spectra in ethanol, ether, ethyl acetate, and in aqueous solutions at pH 7.5 and 1.5. At pH 7.5 light-absorption maxima were at 207, 238 and 306nm (ϵ 25 100, 4920 and 2960 litre·mol⁻¹·cm⁻¹ respectively) and at pH 1.5 they were 208, 246 and 314nm (ϵ 19 840, 6840 and 3060 litre·mol⁻¹·cm⁻¹ respectively). They had the same mobilities on paper in ammonium formate-formic acid (*R*_F 0.60), benzene-acetic acid-water (*R*_F 0.78) and butane-1-ol-pyridine-water (*R*_F 0.30); the spots on paper gave the same blue fluorescence under u.v. light and the same colours on spraying with FeCl₃ and nitroaniline reagent.

Compound II. On the basis of the evidence presented below, compound II is probably *N*¹*N*⁸-bis-(2,3-dihydroxybenzoyl)spermidine (Fig. 2). On paper

chromatography in ammonium formate-formic acid it gave a single spot (R_F 0.40) with a yellow fluorescence under u.v. light. It did not react with ninhydrin, showing that it does not have a primary amine group, but it did react with 1-fluoro-2,4-dinitrobenzene. The ratio E_{350}/E_{390} after the reaction was 0.65. Diethylamine gave a ratio of 0.55, and Dubin (1960) gives values ranging from 0.55 to 0.78 for compounds with secondary amine groups, but lacking primary amine groups. Quantitative analysis, with diethylamine as standard, showed that the compound had $2.0\mu\text{mol}$ of secondary amine/mg. The catechol content was $3.4\mu\text{mol}/\text{mg}$.

Compound II was hydrolysed for 24h in 6M-HCl *in vacuo* at 110°C . Chromatography of the hydrolysate gave one fluorescent spot, which was identical in all respects with 2,3-dihydroxybenzoic acid, and one spot which reacted with ninhydrin. This compound had the same mobilities as spermidine when chromatographed in a number of solvents and electrophoresed at a number of pH values along with standards of putrescine, cadaverine, spermidine, *N*-(3-aminopropyl)-1,3-diaminopropane and spermine. In particular the mobilities of the compound were identical with those of spermidine, but different from those of *N*-(3-aminopropyl)-1,3-diaminopropane, on electrophoresis at pH 4.0 and at pH 6.5, conditions shown by Herbst *et al.* (1958) to separate the homologues *N*-(3-aminopropyl)-1,3-diaminopropane, spermidine and *N*-(4-aminobutyl)-1,4-diaminobutane. *N*-(3-Aminopropyl)-1,5-diaminopentane has been found in *E. coli* by Dion & Cohen (1972); the dansyl derivatives of this compound and of spermidine can be separated by thin-layer chromatography (Dion & Cohen, 1972). Although *N*-(3-aminopropyl)-1,5-diaminopentane was not available as a standard, dansyl-spermidine and the dansyl derivative of the polyamine had identical R_F values in both the solvent systems used by Dion & Cohen (1972). Quantitative determination with ninhydrin showed the presence of $1.9\mu\text{mol}$ of spermidine/mg of compound II. On reaction with 1-fluoro-2,4-dinitrobenzene the hydrolysate gave an E_{350}/E_{390} ratio of 1.34; spermidine gave 1.37, close to the value of 1.45 quoted by Dubin (1960).

From the results presented it is clear that in this compound both primary amine groups of spermidine are in amide linkage with the carboxyl groups of 2,3-dihydroxybenzoic acid, and that the secondary amine group of spermidine is free. Further evidence that compound II contains only spermidine and 2,3-dihydroxybenzoic acid is that it is formed enzymically from these two substrates (see below). *N*¹*N*⁸-Bis-(2,3-dihydroxybenzoyl)spermidine acetate, the probable form of the compound obtained, should have $2.1\mu\text{mol}$ of spermidine and $4.2\mu\text{mol}$ of 2,3-dihydroxybenzoic acid/mg. Elementary analysis showed that compound II was not completely pure, or that it was partially

decomposed. The latter explanation would be consistent with the finding that the content of spermidine was close to the expected value, whereas that of 2,3-dihydroxybenzoic acid was low. 2,3-Dihydroxybenzoic acid is known to be unstable in alkali (Pittard *et al.*, 1961), and compound II was exposed to 2M-NH₃ during purification.

Compound III. On the basis of the evidence presented below, compound III is probably 2-hydroxybenzoyl-*N*-L-threonyl-*N*⁴-[*N*¹*N*⁸-bis-(2,3-dihydroxybenzoyl)]spermidine (Fig. 2). Elementary analysis: found: C, 60.5; H, 5.8; N, 8.7; $\text{C}_{32}\text{H}_{38}\text{N}_4\text{O}_{10}$ requires C, 60.2; H, 6.0; N, 8.8%. Chromatography on paper in a number of solvents gave a single spot which fluoresced blue under u.v. light; in the solvents ammonium formate-formic acid, benzene-acetic acid-water and butan-1-ol-pyridine-water the R_F values were 0.00, 0.92 and 0.78 respectively. Compound III did not migrate on electrophoresis at pH 2. It did not react with ninhydrin or with 1-fluoro-2,4-dinitrobenzene. Compound III was hydrolysed with 6M-HCl at 110°C *in vacuo* for 72h, and by chromatography in a number of solvents, and electrophoresis on paper at a number of pH values, four products were identified; 2,3-dihydroxybenzoic acid, 2-hydroxybenzoic acid, threonine and spermidine. The 2-hydroxybenzoic acid released by hydrolysis and an authentic sample had the same mobilities on paper in ammonium formate-formic acid (R_F 0.64), benzene-acetic acid-water (R_F 0.93) and butan-1-ol-pyridine-water (R_F 0.47); the spots on paper gave the same purple fluorescence under u.v. light and the same colours on spraying with FeCl_3 and nitroaniline reagent. The u.v.-absorption spectrum after elution of the spot from paper with ethanol was identical with that of an ethanol solution of 2-hydroxybenzoic acid. On hydrolysis for 24 or 48h an additional compound, which reacted with ninhydrin, but did not fluoresce under u.v. light, was found. This compound, which on electrophoresis at pH 2 migrated towards the cathode faster than threonine but slower than spermidine, was eluted from the paper, and on further treatment with 6M-HCl at 110°C it broke down completely to give threonine and spermidine.

Compound III before and after hydrolysis was found to contain $2.9\mu\text{mol}$ of catechol/mg. A sample of a 24h hydrolysate was chromatographed on paper, the two fluorescent spots were eluted with ethanol and their spectra were measured. Chromatography and elution of standard mixtures of 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid gave an 84% recovery of both acids. Allowing for this loss, 1mg of compound III contains $2.87\mu\text{mol}$ of 2,3-dihydroxybenzoic acid and $1.29\mu\text{mol}$ of 2-hydroxybenzoic acid. A sample of a 72h hydrolysate was electrophoresed at pH 2, the threonine and spermidine were eluted and their amounts determined by reaction with ninhydrin. It was found that 1mg of compound III contained

1.1 μmol each of threonine and spermidine. By using a standard containing equimolar amounts of threonine and spermidine, the unfractionated hydrolysate was found to contain 1.36 μmol of each/mg. On reaction with 1-fluoro-2,4-dinitrobenzene a 72 h hydrolysate gave an E_{350}/E_{390} ratio of 1.51, exactly the same as that given by a solution containing equimolar amounts of threonine and spermidine.

From these results it is clear that compound III contains 2,3-dihydroxybenzoic acid, 2-hydroxybenzoic acid, threonine and spermidine in a molar ratio of 2:1:1:1. Compound III should then contain 3.14 μmol of 2,3-dihydroxybenzoic acid and 1.57 μmol each of 2-hydroxybenzoic acid, threonine and spermidine/mg. To confirm the composition of compound III, portions of two solutions, one containing 1 mg of compound III and the other containing 3.14 μmol of 2,3-dihydroxybenzoic acid and 1.57 μmol each of 2-hydroxybenzoic acid, threonine and spermidine, were treated with 6M-HCl at 110°C for 24, 48 and 72 h, and the samples were then analysed. The hydrolysates of compound III and of the mixture had identical light-absorption spectra, and gave virtually identical values after reaction with Arnov's nitrate-molybdate reagent, ninhydrin and 1-fluoro-2,4-dinitrobenzene.

A portion (15 mg) of compound III was hydrolysed in 6M-HCl for 72 h and the threonine was isolated by chromatography on Zeo-Karb 225 (H^+) with a gradient of HCl. The threonine was found to be L-threonine. Its specific rotations both in water and in 5M-HCl at 365, 436, 546, 578 and 589 nm were very close to those given by L-threonine (Greenstein & Winitz, 1961). Its rate of oxidation by the L-threonine dehydrogenase of *R. sphaeroides* was measured (see the Experimental section) and was found to be identical with that of L-threonine. This enzyme (Neuberger & Tait, 1962) does not oxidize D-threonine or L-allothreonine and only oxidizes D-allothreonine at about one-half the rate at which it oxidizes L-threonine.

A sample (15 mg) of compound III was hydrolysed

in 6M-HCl for 24 h and the peptide containing threonine and spermidine was isolated by chromatography on Zeo-Karb 225 (H^+) with a gradient of HCl. On reaction with 1-fluoro-2,4-dinitrobenzene this peptide gave a ratio of E_{350}/E_{390} of 2.0, showing that it did not have a free secondary amine group. From this result it is probable that in this dipeptide the carboxyl group of L-threonine is in amide linkage with the secondary amine group of spermidine.

Since in compound II the two 2,3-dihydroxybenzoyl groups are attached to the primary amine groups of spermidine (Fig. 2) and since compound III can be formed from compound II enzymically in the presence of L-threonine and 2-hydroxybenzoic acid (see below), it is likely that in compound III the two 2,3-dihydroxybenzoyl groups are also attached to the primary amine groups of spermidine, that the carboxyl group of L-threonine is in amide linkage with the secondary amine group of spermidine, and that the carboxyl group of 2-hydroxybenzoic acid is in amide linkage with the amino group of L-threonine.

Compound III is soluble in ethanol, ethyl acetate and ether, but relatively insoluble in water except at pH values above 8.5. Light-absorption maxima in ethanol are at 250 and 310 nm (ϵ 26600 and 11000 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ respectively), in aqueous solution at pH 7.5 they are at 208, 246 (shoulder) and 310 nm (ϵ 50000, 18900 and 7700 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ respectively) and at pH 1.5 they are at 210, 253 and 319 nm (ϵ 56900, 27300 and 8200 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ respectively).

The amounts of these three catechol-containing compounds present in the media after anaerobic and aerobic growth for 24 and 48 h are shown in Table 1. After 24 h of anaerobic growth most of the catechol is present as compound II, and only traces of compounds I and III are present. By 48 h the total amount of catechol in anaerobic cultures has increased only slightly, but there are now approximately equal amounts of all three catechols. After 24 h of aerobic

Table 1. *Amounts of catechols excreted by M. denitrificans*

Organisms were grown anaerobically in nitrate medium containing 180 μM -Ca $^{2+}$ and 20 μM -ferric citrate, and aerobically in ammonia medium containing 0.8 μM -ferric citrate (100 ml of medium per flask). Samples were taken at 24 and 48 h and cells were removed by centrifugation. A portion (1 ml) of the supernatants was used to determine the total amount of catechol. The supernatants were adjusted to pH 2 with HCl and extracted with ethyl acetate. The ethyl acetate extracts were extracted with 10% (w/v) NaHCO $_3$. Catechols were assayed in the NaHCO $_3$ extract (compound I), in the solution after extraction with ethyl acetate (compound II), and in the ethyl acetate solution after extraction with NaHCO $_3$ (compound III).

Growth conditions	Time of growth (h)	Catechol concentration (nmol/ml of culture)			
		Total	Compound I	Compound II	Compound III
Anaerobic	24	82	3	54	4
	48	104	27	39	33
Aerobic	24	212	48	105	25
	48	595	108	267	207

growth there are appreciable amounts of all three compounds. During the next 24h the amounts of all three have increased, that of compound III most markedly.

Metal complexes of compounds II and III

On addition of a solution of ferric citrate to a solution of compound III in 0.05M-Tris-HCl buffer, pH8.8, a purple colour, with a light-absorption maximum at 515nm, developed immediately. On titration of a solution of compound III with portions of ferric citrate the E_{515} increased linearly and reached a maximum value when 0.96 μmol of ferric citrate/ μmol of compound III had been added; addition of more ferric citrate did not cause any further increase in E_{515} . Addition of EDTA did not decrease the colour. Compound III (final concn. 0.1mM) was added to 0.13mM solutions of ferric citrate in 0.05M-Tris-HCl buffer, pH8.8, and in 0.5M-EDTA, pH8.2. The rates of increase of E_{515} , and the final absorbances attained, were the same in both solutions. These results show that compound III forms an equimolar ferric complex whose stability constant is much higher than that of the Fe^{3+} -EDTA complex. Compound III also forms complexes when mixed with solutions of Al^{3+} , Cr^{3+} and Co^{2+} as judged from the changes in the u.v.-absorption spectra, and from the fact that subsequent addition of ferric citrate does not give rise to the purple Fe^{3+} -compound III complex.

The ferric complex of compound III was prepared in the following way. To 2ml of water there was added 33 μmol of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1.1 ml of water, and 31.4 μmol of compound III in 1 ml of ethanol. The pH value of the mixture was adjusted to approx. 12 by addition of 3M-NaOH, giving a clear deep-red solution. This solution was stirred for 5 min. Then it was cooled in ice and the pH adjusted to about 3 with 0.5M-HCl, giving a dark-red precipitate. The suspension was centrifuged and the pellet washed twice with 6ml portions of ice-cold water. The solid was dried over NaOH *in vacuo*. The yield was 19mg. This complex was found to contain 73.5 μg of iron/mg, i.e. 91% of the value expected for the unhydrated ferric complex. It is only slightly soluble in water, but is much more soluble at slightly alkaline pH values. In aqueous solution at pH7.4 light-absorption maxima are at 212, 251 (shoulder), 333 and 515nm [ϵ (based on iron content) 75000, 23700, 12700 and 3500 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ respectively] and at pH1.2 they are at 212, 254 and 320nm (ϵ 55800, 30400 and 9400 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ respectively). The spectrum at pH1.2 is very similar to that of compound III at this pH (see above), suggesting that the iron has been removed from the complex.

Addition of small amounts of ferric citrate to an 0.1mM solution of compound II in 0.05M-Tris-HCl

buffer, pH7.7, gave a red solution with a light-absorption maximum at 495nm. The E_{495} increased linearly with increasing amounts of ferric citrate until about 0.5 μmol of ferric citrate had been added/ μmol of compound II. On addition of more ferric citrate the colour changed from red to purple and the absorption maximum shifted to 544nm. The absorbance at this wavelength increased until about 1 μmol of ferric citrate had been added/ μmol of compound II. No further change occurred on addition of more ferric citrate. Addition to this solution of EDTA (5 $\mu\text{mol}/\mu\text{mol}$ of compound II) changed the colour back to red and the absorption maximum to 500nm. Addition of EDTA to a final concentration of 0.15M did not make the solution colourless. These results show that compound II can form a number of complexes with Fe^{3+} , having different stability constants.

Enzymic formation of N¹N⁸-bis-(2,3-dihydroxybenzoyl)spermidine (compound II) and 2-hydroxybenzoyl-N-L-threonyl-N⁴-[N¹N⁸-bis-(2,3-dihydroxybenzoyl)]spermidine (compound III)

Dialysed enzyme extract from *M. denitrificans* grown anaerobically for 32h in low-iron medium was incubated with Mg^{2+} , ATP, dithiothreitol and substrates (Table 2). After the incubation HCl and ethyl acetate were added. After thorough mixing the ethyl acetate layer was removed, its radioactivity measured and a portion chromatographed on paper in ammonium formate-formic acid. Compound II (unlike compound III) is poorly soluble in ethyl acetate, and in assays where it is formed 2ml of ethyl acetate are required to achieve complete extraction from the acidified assay mixture. Incubation with ^{14}C -labelled spermidine and 2,3-dihydroxybenzoic acid gave a single radioactive spot (R_F 0.4) which fluoresced yellow under u.v. light, i.e. properties identical with those of compound II. No radioactivity was extracted into ethyl acetate when ^{14}C -labelled spermidine was incubated alone, and only a small amount was extracted after incubating ^{14}C -labelled spermidine with benzoic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid or 4-hydroxybenzoic acid. When both 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid were incubated with ^{14}C -labelled spermidine there was a small amount of radioactivity at the origin after chromatography in addition to the major spot with R_F 0.4; this pattern was also found when ^{14}C -labelled spermidine was incubated with L-threonine and 2,3-dihydroxybenzoic acid. However, on incubation of ^{14}C -labelled spermidine with L-threonine, 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid, all the radioactivity was in a spot that remained at the origin after chromatography and that fluoresced blue under u.v. light, i.e. properties identical with those of compound III.

Table 2. *Enzymic formation of compounds II and III*

Assay mixtures contained, in a total volume of 0.2 ml, 20 μ mol of Tris-HCl buffer, pH 8.8, 1.5 μ mol of $MgCl_2$, 2 μ mol of dithiothreitol, 1 μ mol of ATP, dialysed enzyme extract (0.9 mg of protein) from cells grown anaerobically in nitrate medium containing 0.8 μ M-ferric citrate for 32 h, and, where stated, 200 nmol of spermidine (when ^{14}C -labelled spermidine was used it had a specific radioactivity of 124 c.p.m./nmol at infinite thinness), 100 nmol of L-threonine (when L- ^{14}C]threonine was used it had a specific radioactivity of 150 c.p.m./nmol at infinite thinness), 100 nmol of 2,3-dihydroxybenzoic acid, 100 nmol of 2-hydroxybenzoic acid and 40 nmol of compound II. After incubation at 37°C for 1 h, 0.1 ml of 0.5 M-HCl and 2 ml of ethyl acetate were added. The tubes were mixed thoroughly and then centrifuged. The ethyl acetate layers were removed. A portion (0.5 ml) was plated and its radioactivity was measured. The rest of the ethyl acetate extract was dried, dissolved in a small volume of ethanol, spotted on paper and chromatographed in 5% (w/v) ammonium formate in 0.5% (v/v) formic acid. Radioactive spots on chromatograms were located by radioautography.

Additions	Radioactivity extracted (nmol of ^{14}C -labelled spermidine or threonine extracted into ethyl acetate)
^{14}C -labelled spermidine	0.0
+2,3-dihydroxybenzoic acid	10.4
+2,3-dihydroxybenzoic acid + 2-hydroxybenzoic acid	7.9
+2-hydroxybenzoic acid	0.2
+L-threonine + 2,3-dihydroxybenzoic acid	10.0
+L-threonine + 2,3-dihydroxybenzoic acid + 2-hydroxybenzoic acid	13.0
L- ^{14}C]Threonine	0.6
+2,3-dihydroxybenzoic acid	1.2
+2-hydroxybenzoic acid	2.1
+spermidine + 2,3-dihydroxybenzoic acid	2.8
+spermidine + 2,3-dihydroxybenzoic acid + 2-hydroxybenzoic acid	15.3
L- ^{14}C]Threonine + compound II	0.5
+compound II + 2,3-dihydroxybenzoic acid	3.0
+compound II + 2-hydroxybenzoic acid	20.5
+compound II + 2,3-dihydroxybenzoic acid + 2-hydroxybenzoic acid	17.8

Only a small amount of radioactivity was extracted into ethyl acetate when enzyme extract was incubated with L- ^{14}C]threonine, and on chromatography it ran as a single spot having an R_F of 0.86. About the same amount of radioactivity was incorporated into this compound in all assays with L- ^{14}C]threonine and enzyme extract, whether or not other substrates were

present. When L- ^{14}C]threonine was incubated with 2,3-dihydroxybenzoic acid or 2-hydroxybenzoic acid slightly more radioactivity was extracted into ethyl acetate, and on chromatography radioactivity was also found in blue fluorescent spots running just in front of 2,3-dihydroxybenzoic acid (R_F 0.56) and 2-hydroxybenzoic acid (R_F 0.64) and having R_F values of 0.69 and 0.76 respectively. These compounds are probably 2,3-dihydroxybenzoyl-*N*-L-threonine and 2-hydroxybenzoyl-*N*-L-threonine. Their mobilities are very similar to that found for 2,3-dihydroxybenzoyl-*N*-L-serine in this solvent by O'Brien *et al.* (1968). Incubation of L- ^{14}C]threonine with spermidine and 2,3-dihydroxybenzoic acid gave only slightly more radioactivity in the ethyl acetate extract, but with 2-hydroxybenzoic acid also, a large amount of radioactivity was extracted. On chromatography this gave a large radioactive spot at the origin which fluoresced blue under u.v. light, i.e. properties identical with those of compound III.

Incubation of compound II with L- ^{14}C]threonine alone or with 2,3-dihydroxybenzoic acid gave only small amounts of ethyl acetate-extractable radioactivity, but on incubation of compound II with L- ^{14}C]threonine and 2-hydroxybenzoic acid there was a marked incorporation into a compound with the chromatographic properties of compound III.

These results show that compound II is formed enzymically from spermidine and 2,3-dihydroxybenzoic acid, and that compound III is formed from compound II, L-threonine and 2-hydroxybenzoic acid, or from spermidine, L-threonine, 2-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid. It can also be seen (Table 2) that about the same amounts of ^{14}C -labelled spermidine and L- ^{14}C]threonine are incorporated into compound III in separate assays, confirming that it contains equimolar amounts of spermidine and L-threonine. The threonyl-spermidine dipeptide isolated after partial hydrolysis of compound III did not form compound III on incubation with 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid. Although from these results it is clear that compound II is an intermediate in the formation of compound III, it is not clear how threonine and 2-hydroxybenzoic acid are added. The formation of small amounts only of a compound that is probably 2-hydroxybenzoyl-*N*-L-threonine in incubations suggests that an enzyme-bound complex of it may be the substrate.

For the formation of compounds II and III in these assays there is an absolute requirement for ATP and a thiol compound, and a partial requirement for Mg^{2+} (see below). D-Threonine and L-serine could not replace L-threonine, nor did they inhibit its incorporation into compound III. *N*-(3-Aminopropyl)-1,3-diaminopropane, a lower homologue of spermidine, could replace spermidine as a substrate, but spermine could not.

Fig. 3. Enzymic formation of compounds II and III with different amounts of enzyme extract

Assays were done, as described in the Experimental section, with dialysed enzyme extract from cells grown anaerobically in nitrate medium containing $0.8 \mu\text{M}$ -ferric citrate. Assays for formation of compound II were done with ^{14}C -labelled spermidine and 2,3-dihydroxybenzoic acid (●); for formation of compound III assays were done either with compound II, L-[^{14}C]threonine and 2-hydroxybenzoic acid (○) or with L-[^{14}C]threonine, spermidine, 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid (△).

Properties of enzyme systems catalysing the formation of compounds II and III

The amounts of compounds II and III formed in assays (for details of assays see the Experimental section) increased linearly with time for up to at least 90 min. With different amounts of enzyme extract (Fig. 3) activities were only linearly proportional to protein concentration at relatively high protein concentrations. The pH optimum for the formation of compound II was 8.8 in 0.1 M-Tris-HCl buffer; at pH 7.3 the activity was only 12% of that at pH 8.8. The pH optimum for the formation of compound III from compound II was 8.6 in 0.1 M-Tris-HCl buffer; at pH 7.3 the activity was 33% of that at pH 8.6.

Compounds II and III were formed in the absence of Mg^{2+} , but their rates of formation were stimulated by a factor of about 1.5 in the presence of 7.5 mM - Mg^{2+} . Little or no formation of compounds II and III occurred in the absence of a thiol compound. Maximum synthesis of both required about 10 mM -dithiothreitol. CoA (0.5 mM) did not stimulate synthesis in the absence or presence of dithiothreitol. There was no formation of compound II or III in the

absence of ATP. In both assays the optimum concentration of ATP was 5 mM .

The formation of compound II was inhibited by 50% by $35 \mu\text{M}$ - Fe^{2+} or $-\text{Fe}^{3+}$, and by $65 \mu\text{M}$ - Al^{3+} . The conversion of compound II into compound III was inhibited by 50% by $110 \mu\text{M}$ - Fe^{2+} or $-\text{Fe}^{3+}$ and by $135 \mu\text{M}$ - Al^{3+} . Cu^{2+} , Co^{2+} and Cr^{3+} were not inhibitory in either assay at concentrations of $200 \mu\text{M}$, whereas at 1 mM only slight inhibitions were found.

Effect of growth conditions on the activities of enzyme systems forming compounds II and III

Dialysed enzyme extracts from cells grown for various times under different conditions were assayed (Figs. 4a, 4b and 4c). Cells grown with sufficient iron did not excrete catechols and enzyme extracts from them did not catalyse the formation of compounds II and III. The specific activities of the enzyme system forming compound II (Fig. 4a) and of the enzyme system that converts compound II into compound III (Fig. 4b) change at different rates under different conditions of growth. The activity of the overall system forming compound III from spermidine, L-threonine, 2-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid is plotted in Fig. 4(c). In extracts from cells grown anaerobically or aerobically in iron-deficient nitrate medium the formation of compound II is the rate-limiting step and in extracts from cells grown aerobically in iron-deficient ammonia medium the conversion of compound II into compound III is the rate-limiting step.

Cells grown anaerobically in nitrate medium containing $20 \mu\text{M}$ -ferric citrate and $180 \mu\text{M}$ - Ca^{2+} had marked enzyme activities for only a short time during growth, and subsequently activities fell to very low values. From 16 to 24 h the growth of this culture increased markedly from 0.27 to 0.57 mg of protein/ml of culture, whereas the growth of the cultures grown in iron-deficient media increased at a much slower rate from about 0.20 to 0.29 mg of protein/ml of culture.

Separation of enzymes involved in the formation of compound II and compound III

Enzyme extract (12.5 mg of protein/ml) from cells grown aerobically for 48 h in iron-deficient ammonia medium was treated with 0.2 vol. of 2% (w/v) protamine sulphate and, after removing the precipitate by centrifugation, a portion of the supernatant was fractionated with solid $(\text{NH}_4)_2\text{SO}_4$ successively at 40, 50, 60 and 80% (w/v) saturation. Each precipitate was collected by centrifugation, dissolved in 0.05 M -Tris-HCl buffer (pH 7.7)– 1 mM -dithiothreitol and dialysed against the same buffer at 4°C overnight. When tested alone these $(\text{NH}_4)_2\text{SO}_4$ fractions had only low activities. However, compound II was

Fig. 4. Effect of growing *M. denitrificans* under different conditions on the specific activities of enzymes forming compounds II and III

Cultures (500 ml) were grown anaerobically and 150 ml cultures were grown aerobically. One culture was harvested at each of the times stated and dialysed enzyme extracts were prepared and assayed as described in the Experimental section. In (a) formation of compound II was assayed, in (b) formation of compound III was assayed with compound II, L-threonine and 2-hydroxybenzoic acid as substrates, and in (c) formation of compound III from L-threonine, spermidine, 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid was assayed. Organisms were grown anaerobically in nitrate medium with $9 \mu\text{M-Ca}^{2+}$ and $0.8 \mu\text{M-ferric citrate}$ (●) and with $180 \mu\text{M-Ca}^{2+}$ and $20 \mu\text{M-ferric citrate}$ (○), and aerobically with $0.8 \mu\text{M-ferric citrate}$ in nitrate medium (Δ) and in ammonia medium (□).

formed when the 0–40% and the 50–60% or the 60–80% (w/v)-satd.-(NH_4) $_2\text{SO}_4$ fractions were assayed together, and compound II was converted into compound III when the 0–40% and 50–60% (w/v)-satd.-(NH_4) $_2\text{SO}_4$ fractions were assayed together. The rest of the protamine sulphate supernatant was chromatographed on DEAE-cellulose (Fig. 5). When tested alone the column fractions had little enzymic activity. Enzyme activities were located by assaying portions (0.1 ml) of the column fractions together with the 0–40% or the 50–60% (w/v)-satd.-(NH_4) $_2\text{SO}_4$ fractions. By combining various column fractions, two peaks of activity, at fractions 108 and 156, for the formation of compound II were found, and two peaks of activity, at fractions 88 and 114, for the conversion of compound II into compound III were found. These results show that at least two proteins are required for the synthesis of compound II, and at least two proteins for its conversion into compound III.

Metabolism of the ferric complex of compound III by iron-deficient cells

It was shown (Tait, 1973) that iron-deficient *M. denitrificans* had a low specific activity of aminolaevulinate synthase, and that the specific activity increased markedly and rapidly on addition of ferric citrate to a culture of iron-deficient organisms. The activity of aminolaevulinate synthase also in-

creased when the ferric complex of compound III was added (Table 3). With low concentrations of ferric citrate or the Fe^{3+} -compound III complex, the activity increased then fell again; with higher amounts the activity increased but did not fall.

These results show that *M. denitrificans* can remove the iron from the very stable Fe^{3+} -compound III complex. There are two possible ways in which it could do this. There could be an enzyme that hydrolyses the amide bonds of the Fe^{3+} -compound III complex resulting in a ferric complex with a lower stability constant. Alternatively there could be an enzyme that reduces the ferric ion in the complex to the ferrous state; this complex would also have a lower stability constant (see the Discussion section).

Incubation of enzyme extracts from iron-deficient organisms with compound III or its ferric complex did not yield any products that fluoresced under u.v. light. When an extract was incubated under N_2 with the Fe^{3+} -compound III complex, 1,10-phenanthroline, succinate and NADH (or NADPH), ferrous 1,10-phenanthroline was formed as shown by an increase in E_{500} (Table 4). The ferrous complex of 1,10-phenanthroline has an ϵ_{500} of $10250 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and the Fe^{3+} -compound (III) complex an ϵ_{500} of about $3500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. As well as an increase in E_{500} during the reaction the colour changed from red to orange. The highest activity (Table 4) occurred in the presence of both succinate and NADH, although there was marked activity with

Fig. 5. Separation of enzymes catalysing the formation of compounds II and III

To 100 ml of enzyme extract (12.5 mg of protein/ml), prepared from cells grown aerobically for 48 h in ammonia medium containing $0.8 \mu\text{M}$ -ferric citrate, there was added 20 ml of 2% (w/v) protamine sulphate. The precipitate was removed by centrifugation and 100 ml of the supernatant was applied to a column (15 cm $\times$ 2 cm) of DEAE-cellulose equilibrated with 0.05 M-Tris-HCl buffer, pH 7.7. Elution, at a rate of 30 ml/h, was performed with a linear gradient made from 500 ml each of 0.05 M-Tris-HCl buffer (pH 7.7)-1 mM-dithiothreitol and 0.4 M-NaCl-0.05 M-Tris-HCl buffer (pH 7.7)-1 mM-dithiothreitol. Fractions (5 ml) were collected. Assays for formation of compound II were done as described in the Experimental section but in a volume of 0.3 ml. Column fractions (0.1 ml) were assayed with 0.1 ml of fraction 156 ($\circ$), or with 0.1 ml of fraction 112 ($\bullet$). Assays for formation of compound III from compound II, L-threonine and 2-hydroxybenzoic acid were done as described in the Experimental section but in a volume of 0.3 ml. Column fractions (0.1 ml) were assayed with 0.1 ml of fraction 118 ($\square$) or with 0.1 ml of fraction 86 ($\blacksquare$).

either one alone. NADPH could replace NADH. Little or no increase in E_{500} occurred in the absence of both succinate and NADH, or when they were present but enzyme extract, Fe^{3+} -compound III complex or 1,10-phenanthroline was omitted. There was some activity when the complete assay mixture was incubated in tubes open to the air, but this was considerably less than the activity under N_2 . The E_{500} increased linearly with time for up to 2 h, and the increase in E_{500} was linearly proportional to the amount of enzyme extract added up to about 3 mg of protein. There was marked activity over the pH range 5.5-8.8, with an optimum at about pH 6.5. It was found that the concentrations of succinate, NADH and 1,10-phenanthroline used in Table 4 gave the highest activities. Comparable activities were given by enzyme extracts before and after dialysis

Table 3. Effect on aminolaevulinate synthase activity of adding Fe^{3+} -compound III complex and ferric citrate to iron-deficient cultures

Cultures were grown anaerobically for 24 h in nitrate medium containing $0.8 \mu\text{M}$ -ferric citrate. At 24 h Fe^{3+} -compound III complex or ferric citrate were added. Samples were taken at the times stated and aminolaevulinate synthase activity was measured.

Additions ($\mu\text{mol/litre}$ of culture)	Time	Aminolaevulinate synthase activity (nmol of aminolaevulinate/h per mg of protein)		
		24 h	25 h	26 h
None	...	7.0	9.6	13.8
Ferric citrate (0.48)		13.3	44.1	20.8
Fe^{3+} -compound III (0.58)		14.2	36.2	18.4
Ferric citrate (0.96)		14.6	51.4	45.9
Fe^{3+} -compound III (1.5)		9.5	44.0	33.6
Ferric citrate (2.4)		12.6	45.7	48.5
Fe^{3+} -compound III (2.9)		11.1	47.5	45.0

Table 4. Enzymic formation of ferrous 1,10-phenanthroline from Fe^{3+} -compound III

Assay mixtures contained, in a total volume of 0.5 ml, $50 \mu\text{mol}$ of phosphate buffer, pH 6.5 (KH_2PO_4 -NaOH; $50 \mu\text{mol}$ of phosphate), $10 \mu\text{mol}$ of sodium succinate, $0.5 \mu\text{mol}$ of NADH, $1 \mu\text{mol}$ of 1,10-phenanthroline, 60 nmol of ferric compound III and dialysed enzyme extract (1.44 mg of protein) from cells grown anaerobically for 32 h in nitrate medium containing $0.8 \mu\text{M}$ -ferric citrate. Tubes were flushed with N_2 , sealed with Parafilm and incubated at 37°C for 1 h. Control tubes containing the same constituents were incubated at 0°C . E_{500} was measured in micro-cuvettes with a 1 cm path-length. E_{500} of controls were subtracted from E_{500} of experimental tubes.

Omissions	E_{500}
1,10-Phenanthroline	0.000
Fe^{3+} -compound III	0.112
Enzyme extract	0.107
Succinate and NADH	0.190
NADH	0.454
Succinate	0.526
None	0.623

against 0.05 M-Tris-HCl buffer, pH 7.7, without or with 1 mM-dithiothreitol or 2-mercaptoethanol. Extracts that had been dialysed in the absence of a thiol compound lost their activity more rapidly on storage.

Enzyme extract was treated with 0.2 vol. of 2% (w/v) protamine sulphate, and after removing the precipitate by centrifugation, the supernatant was fractionated with solid $(\text{NH}_4)_2\text{SO}_4$ successively at 40, 50, 60 and 80% (w/v) saturation. Each precipitate was collected by centrifugation, dissolved in 0.05 M-

Tris-HCl buffer (pH 7.7)–1 mM-2-mercaptoethanol and dialysed against the same buffer. When assayed individually none of the $(\text{NH}_4)_2\text{SO}_4$ fractions had a marked activity to form ferrous 1,10-phenanthroline. However, high activity was obtained when the 0–40% and the 50–60% (w/v) satd. $(\text{NH}_4)_2\text{SO}_4$ fractions were assayed together.

Discussion

The three catechol-containing compounds that accumulate in the medium when *M. denitrificans* is grown under conditions of iron deficiency have been identified as 2,3-dihydroxybenzoic acid, N^1N^8 -bis-(2,3-dihydroxybenzoyl)spermidine and 2-hydroxybenzoyl-*N*-L-threonyl- N^4 -[N^1N^8 -bis-(2,3-dihydroxybenzoyl)spermidine. Of these compounds have not been described previously; it is formed by a number of micro-organisms when they are grown under conditions of iron deficiency (see the Introduction). Spermidine is present in many micro-organisms, and it is thought to play a number of important roles in metabolism (Bachrach, 1973). Siderochromes containing spermidine have not been described before, although the basic amino acid L-lysine is a constituent of the iron-binding compound N^2N^6 -bis-(2,3-dihydroxybenzoyl)-L-lysine formed by *A. vinelandii* (Corbin & Bulen, 1969). The presence of both 2,3-dihydroxybenzoic acid and 2-hydroxybenzoic acid (salicylic acid) in a siderochrome is also novel. 2-Hydroxybenzoic acid is present in the mycobactins, iron-binding compounds formed by Mycobacteria, but in them the other ligands are two hydroxamic acid groups and a tertiary nitrogen atom (Neilands, 1972).

A number of the findings reported in the present paper show that the catechols excreted by *M. denitrificans* are involved in iron transport. These compounds are only produced in detectable quantities when cells are grown in media deficient in iron, the enzymes for the formation of compounds II and III can only be detected in extracts from iron-deficient cells, on addition of the Fe^{3+} -compound III complex to an iron-deficient culture the activity of aminolaevulinic synthase increases, and extracts of iron-deficient cells catalyse the reduction of iron in the Fe^{3+} -compound III complex to the ferrous state.

Compound III forms a red-purple equimolar complex with Fe^{3+} . Construction of a space-filling molecular model of compound III shows that all five phenolic hydroxyl groups can bind to the Fe^{3+} ion, but that the hydroxyl group of the L-threonyl residue cannot. The structure of the model of the Fe^{3+} -compound III complex, and in particular the arrangement in space of the aromatic rings, is very similar to that of the molecular model of Fe^{3+} -enterochelin published by O'Brien & Gibson (1970)

(see also Llinas *et al.* 1973) where all six phenolic hydroxyl groups bind to the Fe^{3+} ion (Pollack & Neilands, 1970). The formation of the ferric complex of compound III proceeds at the same rapid rate in the presence and absence of 0.5 M-EDTA, showing that its stability constant, like that of Fe^{3+} -enterochelin, is higher than that of Fe^{3+} -EDTA, whose stability constant is 10^{25} (O'Brien *et al.*, 1971).

In order for siderochromes to function as iron-transport compounds, cells must have a mechanism for release of the iron from the ferric-siderochrome complex after it has been transported into the cell. Direct transfer of iron to other ligands is unlikely because of the very high stability of the complex. In *E. coli* this is achieved by an esterase that hydrolyses Fe^{3+} -enterochelin to yield 2,3-dihydroxybenzoyl-*N*-L-serine and iron complexes of it (O'Brien *et al.*, 1971). The stability constants of the ferric complexes of 2,3-dihydroxybenzoylserine are considerably lower than that of Fe^{3+} -enterochelin (O'Brien *et al.*, 1971) and the iron can now be removed and complexed with protoporphyrin (Porra *et al.*, 1972) and proteins. In *Mycobacterium smegmatis*, on the other hand, there is an enzyme that reduces the iron in ferric-mycobactin to the ferrous state under anaerobic conditions in the presence of NADH (Ratledge, 1971). Since mycobactin, like most microbial siderochromes, binds ferrous iron much less firmly than ferric iron (Neilands, 1972) the iron can now be removed and utilized by the cell. Extracts of iron-deficient *M. denitrificans* did not cleave the amide bonds of compound III or its ferric complex, but did catalyse the reduction of iron in the ferric complex of compound III. The mechanism of this reaction is not known, but preliminary experiments show that more than one protein is involved.

Cultures of *M. denitrificans* grown anaerobically in nitrate medium with 20 μM -iron citrate show symptoms of iron deficiency if the medium also contains a high concentration of Ca^{2+} (180 μM). Under this condition of growth catechols are excreted, and for a short period during growth the organisms contain the enzymes that catalyse the synthesis of compounds II and III (Fig. 4). It seems likely that Ca^{2+} interferes in some way with the uptake of ferric citrate and that the cells respond to this apparent deficiency in iron by making and excreting siderochromes. That this leads to uptake of iron, probably as the Fe^{3+} -compound III complex, is shown by the rapid increase in growth that occurs between 16 and 24 h, and by the rapid fall in the activities of the enzymes that catalyse the formation of compounds II and III (Fig. 4).

The enzyme system catalysing the synthesis of compound III seems to be similar to the one catalysing the synthesis of enterochelin from 2,3-dihydroxybenzoic acid and L-serine in *E. coli* (Bryce & Brot, 1972; Bryce, 1973) in that a number of proteins

are involved, a high concentration of a thiol compound has to be present in assay mixtures, and ATP is the only cofactor required. From their results Bryce & Brot (1972) conclude that enterochelin is formed in the following way. One enzyme binds L-serine through a thioester linkage in an ATP-mediated reaction, a second enzyme forms a complex with 2,3-dihydroxybenzoic acid and AMP, and their interaction produces a 2,3-dihydroxybenzoyl-*N*-L-seryl-enzyme complex. This complex is then acted on by a third enzyme yielding cyclotris-(2,3-dihydroxybenzoyl-*N*-L-seryl) (enterochelin). Free 2,3-dihydroxybenzoylserine is not an intermediate (see also O'Brien *et al.*, 1971). The formation of compound II from spermidine and 2,3-dihydroxybenzoic acid requires at least two enzymes. It seems possible that one is required to activate the carboxyl group of 2,3-dihydroxybenzoic acid and the other to catalyse amide bond formation between this activated carboxyl group and the primary amine groups of spermidine. It is not clear how compound II is converted into compound III. Since very much less 2-hydroxybenzoylthreonine is formed enzymically in the absence of compound II than is incorporated into compound III in the presence of compound II (Table 2) it seems likely that a 2-hydroxybenzoylthreonyl-enzyme complex is the substrate that reacts with compound II. If the mechanism of the reaction is similar to that found for enterochelin synthesis conversion of compound II into compound III might be expected to require three enzymes, two to form the 2-hydroxybenzoylthreonyl-enzyme complex and one to catalyse its reaction with the secondary amine group of compound II.

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