Pigments of *Pseudomonas* Species. Part V.[1] Biosynthesis of Pyocyanin and the Pigments of *Ps. aureofaciens* †

By **M. E. Flood, R. B. Herbert,** and **F. G. Holliman,*** Department of Organic Chemistry, The University of Leeds, Leeds LS2 9JT

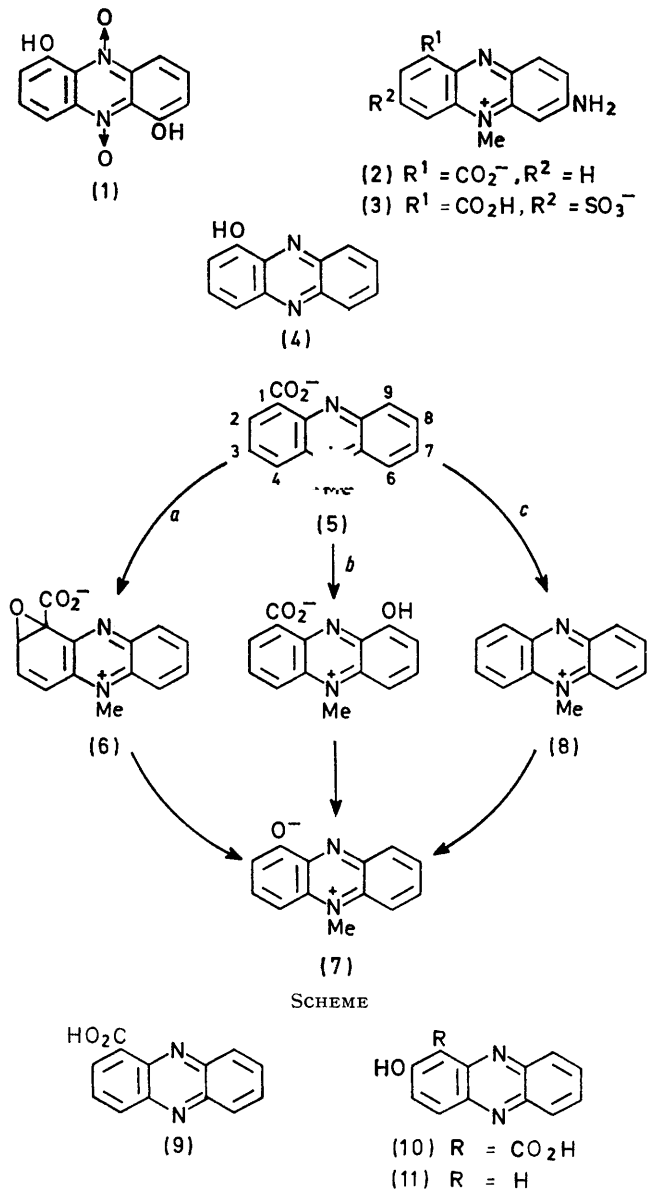
Tracer experiments have shown that phenazine-1-carboxylic acid and its 5-methyl betaine are incorporated into pyocyanin by *Ps. aeruginosa* by decarboxylative hydroxylation. Phenazine-1,6-dicarboxylic acid is not incorporated. 2-Hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine are derived from phenazine-1-carboxylic acid in *Ps. aureofaciens* but not from 5-methylphenazinium-1-carboxylate.

THE phenazine ring system is common to some 30 microbial metabolites. Considerable effort has been expended on discovering how this skeleton is constructed by micro-organisms. It has been suggested that formation takes place by the coupling of two anthranilic acid units.[2] Although anthranilic acid is used in the biosynthesis of secondary metabolites in plants [3,4] and micro-organisms,[4] there is only sparse evidence [2] to support a role for it in the biosynthesis of the phenazine ring system. On the other hand, two shikimic acid molecules are implicated in the biosynthetic pathway.[5]

With regard to the later stages in the biosynthesis of these metabolites it has been shown that phenazine-1,6-diol and its 5-oxide are efficiently incorporated into iodinin (1).[6] Also, 5-methylphenazinium-1-carboxylate (5) has been converted by *Pseudomonas aeruginosa* into aeruginosin A (2).[1] On the other hand, 1-hydroxyphenazine (4) is not a precursor of pyocyanin (7).[7]

Pyocyanin is produced by *Ps. aeruginosa*, alternative strains of which produce the aeruginosins, A (2) [8] and B (3).[9] The production of these three pigments by different strains of the same organism suggested that their biosynthetic pathways might be similar and, in particular, that the betaine (5) might be a precursor for pyocyanin (7).[10] There are three ways in which the transformation (5) ⟶ (7) could take place. The first involves hydroxylative decarboxylation (Scheme, path *a*). The second and third (paths *b* and *c*) involve separate steps of decarboxylation and hydroxylation.

In order to distinguish between these three paths, appropriately deuteriated precursors were used in preference to combined $^3H^{14}C$ labels, as incorporations were expected to be sufficiently high for mass spectral estimation and deuteriated compounds could be much more conveniently synthesized. The appropriately labelled betaine (5) was therefore 6,7,8,9-tetradeuterio-5-methylphenazinium-1-carboxylate: path *a* would result in retention of all the deuterium atoms whereas



SCHEME

path *b* would result in loss of one. Path *c*, proceeding through species (8) which is symmetrical, would give

† Preliminary communication, M. E. Flood, R. B. Herbert, and F. G. Holliman, *Chem. Comm.*, 1970, 1514.

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[6] N. N. Gerber, *Biochemistry*, 1967, **6**, 2701.

[7] L. H. Frank and R. D. DeMoss, *J. Bacteriol.*, 1959, **77**, 776.

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tri- and tetra-deuterio-(7) in equal proportions unless (8) only existed bound to an enzyme surface.

6,7,8,9-Tetradeuterio-5-methylphenazinium-1-carboxylate was synthesized *via* the same reaction sequence as the non-deuteriated material,[1] beginning with 2,3,4,5,6-pentadeuterioaniline.[11] 6,7,8,9-Tetradeuteriophenazine-1-carboxylic acid [as (9)] obtained as an intermediate in this synthetic sequence was decarboxylated to give 1,2,3,4-tetradeuteriophenazine. *N*-Methylation[12] of the deuteriophenazine gave 1,2,3,4-tetradeuterio-5-methylphenazinium methosulphate [as (8) methosulphate], whereas photolysis in acid solution gave a mixture of equal quantities of 1,2,3-trideuterio-4-hydroxyphenazine and 1,2,3,4-tetradeuterio-6-hydroxyphenazine.

Samples of these deuteriated materials [as (4), (5), (8) (methosulphate), and (9)] were administered to pyocyanin-producing strains of *Ps. aeruginosa* over the period of pigment production. Pyocyanin did not give a satisfactory mass spectrum; the sample of the pigment isolated from each experiment was therefore dequaternized in alkali to 1-hydroxyphenazine, the mass spectrum of which is simple in the high mass region where for the undeuteriated material, two prominent peaks are found at *m/e* 196 (M^+) and 168 ($M^+ - CO$). Dequaternization of 2,3,4-trideuteriopyocyanin under similar conditions gave 1,2,3-trideuterio-4-hydroxyphenazine,[13] showing that the conditions do not lead to loss of deuterium. The results appear in Table 1 and show that compounds (5), (8), and (9) are precursors for pyocyanin (7).

TABLE 1

Compound administered to *Ps. aeruginosa*	Amount administered (mg)	Pyocyanin (mg)	Deuterium content (%)	Incorporation (%)
6,7,8,9-Tetradeuterio-5-methylphenazinium-1-carboxylate	24·4	17·3	2·6; $^2H_4$ only	2·4
	24·0	54·2	3·8; $^2H_4$ only	11·0
6,7,8,9-Tetradeuteriophenazine-1-carboxylic acid	22·5	15·9	1·8; $^2H_4$ only	1·4
	19·3	51·7	4·1; $^2H_4$ only	11·7
1,2,3,4-Tetradeuterio-5-methylphenazinium methosulphate	18·7	26·4	0·3; *ca.* equal amounts of $^2H_3$ and $^2H_4$	0·7
Mixture of 1,2,3-trideuterio-4-hydroxyphenazine and 1,2,3,4-tetradeuterio-6-hydroxyphenazine	24·2	20·6	0	0

As both of the tetradeuterio-samples of (5) and (9) are incorporated into pyocyanin without loss of deuterium, the hydroxylation is a specific decarboxylative reaction. This specificity, together with the high level of incorporation of these two compounds into pyocyanin, demonstrates that they are true precursors, and since 1-hydroxyphenazine was not incorporated, in confirmation of the earlier finding,[7] the sequence must be (9) ⟶ (5) ⟶ pyocyanin (7). Further, the specificity of the hydroxylation reaction demonstrates that path *b* is not utilized, and path *c* can be followed only if (8) remains enzyme-bound and thus unsymmetrical; path *a* is in accord woth the results. The incorporation found for (8) methosulphate is consistent with its acting as a convenient substrate for hydroxylation.

Arene oxides are important as intermediates in substrate hydroxylation by mixed function oxidases.[14] Thus species (6) may be considered to be the initial product of the hydroxylation of (5). This is an intermediate which could readily collapse to pyocyanin. Such a reaction sequence has been proposed for the biological conversion of *p*-aminobenzoic acid into *p*-aminophenol.[15]

Pseudomonas aureofaciens produces an abundance of 1-carboxyphenazine together with small amounts of 2-hydroxyphenazine-1-carboxylic acid (10)[16] and 2-hydroxyphenazine (11).[17] 2-Hydroxyphenazine-1-carboxylic acid could arise from species (6) by the alternative opening of the oxide ring, involving loss of a proton rather than carbon dioxide, followed by dequaternization.

In order to test this hypothesis the deuterio-compounds [as (5) and (9)] were administered to *Ps. aureofaciens* under conditions similar to those used with *Ps. aeruginosa*, and the three metabolites were isolated. The two carboxylic acids were best examined in the mass spectrometer as their methyl esters: 2-hydroxyphenazine-1-carboxylic acid gave a poor spectrum and that of phenazine-1-carboxylic acid showed a peak at $M^+ + 2$, of intensity comparable to that of M^+, attributable to thermal formation of the presumably more volatile dihydrophenazine; the intensity of this peak was dependent on the temperature of the mass spectrometer source. The presence of this peak made accurate determination of the deuterium content of

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[15] N. H. Sloane and K. G. Untch, *Biochemistry*, 1964, **3**, 1160; G. A. Hamilton, *Adv. Enzymol.*, 1969, **32**, 88.

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[17] M. E. Levitch and P. Rietz, *Biochemistry*, 1966, **5**, 689.

the deuteriated phenazine-1-carboxylic acid difficult. Methyl phenazine-1-carboxylate, however, gave no $M^+ + 2$ peak.

It was found that whilst phenazine-1-carboxylic acid was utilized in the biosynthesis of compounds (10) and (11), 5-methylphenazinium-1-carboxylate was not incorporated (nor dequaternized to 1-carboxyphenazine); the results for phenazine-1-carboxylic acid appear in Table 2. Thus 2-hydroxyphenazine and 2-hydroxyphenazine-1-carboxylic acid are obtained from phenazine-1-carboxylic acid directly and the respective levels of incorporation are in accord with the sequence (9) ⟶ (10) ⟶ (11). As phenazine-1-carboxylic acid is so much more abundant than the other and so deuterium content was estimated from the mass spectrum of the dimethyl ester, which did not show an $M^+ + 2$ peak.

2,4-Dideuteriophenazine-1,6-dicarboxylic acid was administered over the pigment-producing period to *Ps. aeruginosa*. It was not incorporated into pyocyanin (7). A similar result was obtained when the acid was administered over the early period of culture growth and the pyocyanin isolated at the usual stage of pigment production. Whilst negative results must always be treated with caution, it seems as if phenazine-1,6-dicarboxylic acid is not a precursor for pyocyanin and thus, perhaps, not for other metabolites derived from phenazine-1-carboxylic acid.

TABLE 2

6,7,8,9-Tetradeuteriophenazine-1-carboxylic acid

Amount administered (mg)	Amount isolated at the end of the experiment (mg)	Deuterium content (%) [a]	Amount of deuteriated acid used by organism (mg) [b]	Metabolite isolated	Amount isolated (mg)	Deuterium content (%)	Incorporation (%) [c]
52·9	203·9	17·6 [d]	17·0	(10)	26·7	11·2 [d]	16·4
				(11)	5·6	5·9 [d]	2·2
66·8	69·0	64·3 [d]	22·4	(10)	4·6	40·3 [d]	7·7
				(11)	1·1	26·7 [d]	1·5

[a] Calculated from the mass spectrum of the methyl ester. [b] Calculated from the previous three columns. [c] Calculated from the amount of deuteriated phenazine-1-carboxylic acid used by the organism. [d] Material contained four deuterium atoms only.

two pigments, the hydroxylation reaction, which may include an intermediate analogous to (6), is inefficient. This is in contrast to the biosynthetic sequence which leads to pyocyanin (7). Certainly, phenazines are much less reactive *in vitro* than phenazinium salts towards nucleophiles.

When the phenazine ring is formed biologically from two shikimic acid-derived units the initial product could well be phenazine-1,6-dicarboxylic acid, itself a microbial metabolite.[18] The intermediacy here of this acid (or of a reduced counterpart), which can conceivably be converted into more highly oxidized phenazines by processes involving hydroxylation and/or decarboxylation, seems more likely than the suggestion [5a] that the intermediate is 4,9-dihydroxyphenazine-1,6-dicarboxylic acid, which would, in the case of most phenazine metabolites, necessitate conversion into less hydroxylated systems. It is possible then that phenazine-1-carboxylic acid [and metabolites like (7), (10), and (11) derived from it] might be biosynthesized from phenazine-1,6-dicarboxylic acid.

Phenazine-1,6-dicarboxylic acid was produced along with phenazine-1,8-dicarboxylic acid when 2-(3-carboxyanilino)-3-nitrobenzoic acid (prepared from 2-bromo-3-nitrobenzoic acid and 3-aminobenzoic acid) was cyclized by the alkaline sodium borohydride method.[19] 2,4-Dideuteriophenazine-1,6-dicarboxylic acid was prepared from 3-amino-2,4,6-trideuteriobenzoic acid (obtained by repeated acid-catalysed exchange on 3-aminobenzoic acid; exchange was incomplete). Like phenazine-1-carboxylic acid, phenazine-1,6-dicarboxylic acid gave an $M^+ + 2$ peak, together with an M^+ peak,

We are currently investigating the possibility of phenazine-1,6-dicarboxylic acid being a precursor for other metabolites and attempting to clarify the mechanisms of hydroxylation.

EXPERIMENTAL

Mass spectra were obtained on an A.E.I. MS 902 instrument with direct insertion of the sample. M.p.s were determined on a Kofler hot-stage apparatus.

2-(3-Carboxyanilino)-3-nitrobenzoic Acid.—2-Bromo-3-nitrobenzoic acid (1·0 g), 3-aminobenzoic acid (0·86 g), anhydrous potassium carbonate (0·9 g), and copper bronze (0·05 g) were heated in refluxing ethanol (10 ml) for 26 h. The ethanol was evaporated off and the residue was dissolved in water. The solution was filtered and acidified. The yellow precipitate yielded the *acid* (775 mg, 63%), m.p. 287—290° (from ethanol); $\lambda_{max.}$ 255 and 322 nm; $\nu_{max.}$ (Nujol) 3310, 1690, and 1671 cm^{-1}; *m/e* 302 (M^+), 284 ($M^+ - 18$), and 258 ($M^+ - 44$) (Found: C, 55·75; H, 3·5; N, 9·3. $C_{14}H_{10}N_2O_6$ requires C, 55·65; H, 3·3; N, 9·3%); *methyl ester* (diazomethane in methanol), m.p. 107·5°—109·5° (from methanol), $\nu_{max.}$ (Nujol) 3230, 1700, and 1678 cm^{-1}; $\lambda_{max.}$ 250 and 325 nm (Found: C, 58·45; H, 4·4; N, 8·1. $C_{16}H_{14}N_2O_6$ requires C, 58·2; H, 4·25; N, 8·5%).

Cyclization of 2-(3-Carboxyanilino)-3-nitrobenzoic Acid.—A solution of the anilinobenzoic acid (600 mg), sodium borohydride (390 mg), and sodium ethoxide [from sodium (556 mg)] in absolute ethanol (15 ml) was heated under reflux for 30 h. Sufficient water (50 ml) was added to dissolve the precipitate which had formed and the ethanol

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[19] S. R. Challand, R. B. Herbert, and F. G. Holliman, *Chem. Comm.*, 1970, 1423.

was removed under reduced pressure. The aqueous solution was acidified to give a dark green precipitate which was collected by centrifugation and dried. Recrystallization from dimethylformamide gave phenazine-1,6-dicarboxylic acid (186 mg, 35%), m.p. >290°, $\nu_{max.}$ (Nujol) 1724 cm^{-1}; $\lambda_{max.}$ (EtOH) 253 and 368 nm; *m/e* 270·06346 (Calc. for $C_{14}H_8N_2O_4$,2H: 270·06405, Δ 2·2 p.p.m.), 252 ($C_{14}H_8N_2O_3$), 234, 224 ($C_{13}H_8N_2O_2$), 206, 180, and 179; τ ($CF_3{\cdot}CO_2H$) 0·67 (2H, dd, *J* 2 and 7·5 Hz), 0·97 (2H, dd, *J* 2 and 9 Hz), and 1·45 (2H, dd, *J* 7·5 and 9 Hz) (Found: C, 62·45; H, 3·25; N, 10·6. Calc. for $C_{14}H_8N_2O_4$: C, 62·7; H, 2·85; N, 10·45%); dimethyl ester (diazomethane in dimethylformamide), m.p. 230—232° (lit.,[18] 229—230°), $\lambda_{max.}$ (EtOH) 247·5 and 364 nm; *m/e* 296 (M^+), 281 (M^+ − 15), 265 (M^+ − 31), and 238 (M^+ − 58); τ ($CF_3{\cdot}CO_2H$) 0·89 (2H, dd, *J* 2 and 6·5 Hz), 1·02 (2H, dd, *J* 2 and 9 Hz), 1·47 (2H, dd, *J* 6·5 and 9 Hz), and 5·66 (6H, s). (Found: C, 64·8; H, 4·45; N, 9·1. Calc. for $C_{16}H_{12}N_2O_4$: C, 64·9; H, 4·05; N, 9·45%).

The mother liquor from the recrystallization of phenazine-1,6-dicarboxylic acid was treated with ethereal diazomethane for 1 hr. Water (20 ml) was added and the mixture was extracted with chloroform. The extract was dried and chromatographed on a column of Kieselgel G (benzene–20% ethyl acetate). *Dimethyl phenazine-1,8-dicarboxylate* was isolated as the major fraction (148 mg), m.p. 159·5—160·5° (from ethanol), $\nu_{max.}$ (Nujol) 1716 cm^{-1}; $\lambda_{max.}$ (EtOH) 259 and 368 nm; *m/e* 296 (M^+), 265 (M^+ − 31), and 238 (M^+ − 58); τ ($CDCl_3$). 0·97 (1H, s), 1·55—1·75 (4H, unresolved), 1·93 (1H, d, *J* 2 Hz), 2·17 (1H, t, *J* 8 and 9 Hz), 6·87 (3H, s), and 6·97 (3H, s) (Found: C, 64·7; H, 4·45; N, 9·1. $C_{16}H_{12}N_2O_4$ requires C, 64·9; H, 4·05; N, 9·45%).

3-Amino-2,4,6-trideuteriobenzoic Acid.—3-Aminobenzoic acid hydrochloride (0·5 g) in deuterium oxide (10 ml) was heated at 100° in a sealed tube for 24 h. The mixture was taken to dryness and more deuterium oxide (10 ml) was added. The process of adding fresh deuterium oxide, heating, and evaporating to dryness was repeated four times. The 3-aminobenzoic acid showed *m/e* 140 and 139 (M^+); 67% $^2H_3$, 33% $^2H_2$.

2-(3-Carboxy-2,4,6-trideuterioanilino)-3-nitrobenzoic Acid.—This material had m.p. 286—288°, *m/e* 305 and 304 (M^+); isotopic purity 66% $^2H_3$, 34% $^2H_2$.

2,4-Dideuteriophenazine-1,6-dicarboxylic Acid.—This substance showed $\nu_{max.}$ (Nujol) 1724 cm^{-1}; τ ($CF_3{\cdot}CO_2H$) 0·60 (1H, d, *J* 7·5 Hz), 0·94 (1H, d, *J* 9 Hz), 1·37 (1H, dd, *J* 7·5 and 9 Hz), and 1·37 (1H, s); only the signals due to the dideuterio-species were distinguishable; dimethyl ester, *m/e* 296 and 295 (M^+); isotopic purity 68% $^2H_2$, 32% $^2H_1$.

3-Nitro-2-(2,3,4,5,6-pentadeuterioanilino)benzoic Acid.—This compound had m.p. 195—196·5°; $\nu_{max.}$ (Nujol) 3400 and 1660 cm^{-1}; *m/e* 263 (M^+), 245 (M^+ − 18, *m** 228), 227, and 215; 97% $^2H_5$; τ (Me_2CO) 1·70 (1H, dd, *J* 1·5 and 7·5 Hz), 1·97 (1H, dd, *J* 1·5 and 8·5 Hz), and 2·93 (1H, t, *J* 8 Hz).

6,7,8,9-Tetradeuteriophenazine-1-carboxylic Acid.—This had m.p. 242—243°; $\nu_{max.}$ (Nujol) 1735 cm^{-1}; *m/e* 230 (M^+ + 2), 228 (M^+), and 184 (M^+ − 44), 97% $^2H_4$; τ ($CF_3{\cdot}CO_2H$) 0·63 (1H, dd, *J* 1·5 and 7·5 Hz), 0·93 (1H, dd, *J* 1·5 and 9 Hz), and 1·55 (1H, dd, *J* 7·5 and 9 Hz).

Methyl 6,7,8,9-Tetradeuteriophenazine-1-carboxylate.—This had m.p. 120·5—122°; $\nu_{max.}$ (Nujol) 1715 cm^{-1}; *m/e* 242 (M^+), 227 (M^+ − 15), 211 (M^+ − 31), 184, and 183 (211 − 28, *m** 159), 97% $^2H_4$; τ ($CDCl_3$) 1·63 (1H, dd, *J* 2 and 8 Hz), 1·91 (1H, dd, *J* 2 and 7·5 Hz), 2·21 (1H, dd, *J* 7·5 and 8 Hz), and 5·9 (3H, s).

1-Carboxy-6,7,8,9-tetradeuterio-5-methylphenazinium Chloride.—The compound gave a single spot, identical with that from a sample of non-deuteriated material, on chromatography (Whatman no. 1 paper; 4 : 1 butanol and conc. hydrochloric acid saturated with water). Dequaternization gave *deuteriated phenazine-1-carboxylic acid*, identical with a non-deuteriated specimen by t.l.c. (Kieselgel G; 10% MeOH–$CHCl_3$). Isotopic purity 97% $^2H_4$ by mass spectrometry.

1,2,3,4-Tetradeuteriophenazine.—A suspension of 6,7,8,9-tetradeuteriophenazine-1-carboxylic acid (64 mg) in quinoline (1 ml) was refluxed with copper powder (20 mg) for 10 min. The mixture was filtered and the residue was washed with chloroform. The combined solutions were extracted with aqueous sodium hydroxide (0·1N) then water and dried. Quinoline was removed at 0·1 mmHg (bath temp. 65°). The residue was sublimed (120° and 0·1 mmHg). The sublimate was recrystallized from ethanol–water to give the *deuteriophenazine* (33·5 mg), m.p. 174—176°, *m/e* 184 (M^+), 97% $^2H_4$.

1,2,3-Trideuterio-4-hydroxyphenazine and 1,2,3,4-Tetradeuterio-6-hydroxyphenazine.— 1,2,3,4-Tetradeuteriophenazine (33 mg) was photolysed (Hanovia UVS 500A; quartz jacket) in hydrochloric acid (1N; 400 ml) for 30 min. The solution was extracted with chloroform. The chloroform solution was extracted with aqueous sodium hydroxide (0·1N). The aqueous extract was then acidified and extracted with chloroform. This chloroform extract was dried and evaporated. The residue was sublimed (120° and 0·1 mmHg) to give the 1-hydroxyphenazine (30 mg), m.p. 156—157°; *m/e* 200, 199 (M^+), 172, and 171 (M^+ − 28), 46% $^2H_4$, 54% $^2H_3$.

1,2,3,4-Tetradeuterio-5-methylphenazinium methosulphate had m.p. 149—149·5° (decomp.); *m/e* 200 ($C_{13}H_7D_4N_2$,H) and 184 ($C_{12}H_4D_4N_2$); 97% $^2H_4$.

Pigment Production and Isolation.—(A) *Pyocyanin.* A pyocyanin-producing strain of *Pseudomonas aeruginosa* was grown in liquid culture[20] in Glaxo flasks (200 ml per flask) at 37°. The cultures were extracted with chloroform when the blue pigmentation was judged to be at a maximum (*ca.* 5 days). The chloroform extract was dried, evaporated to small volume, and subjected to preparative t.l.c. ($CHCl_3$–10% MeOH). The blue band corresponding to pyocyanin was eluted ($CHCl_3$–5% MeOH). The solvent was removed and the residue was recrystallized from water; m.p. 132—133° (lit.,[21] 133°); yield 15—55 mg l^{-1}. Dequaternization (1N aq. NaOH; 100°; 30 min) gave 1-hydroxyphenazine, m.p. 156—157·5° (lit.,[22] 158°).

(B) *The pigments of* Ps. aureofaciens. *Ps. aureofaciens* was cultured as previously described.[23] The pigments were isolated 4 days after innoculation of the growth medium. The cultures were acidified to pH 4 and extracted thoroughly with chloroform. (Emulsions were broken by centrifugation.) The chloroform extract was

[20] M. O. Burton, J. J. R. Campbell, and B. A. Eagles, *Canad. J. Res.*, 1947, **26***c*, 15.
[21] A. R. Surrey, *Org. Synth.*, 1946, **26**, 86.
[22] F. Wrede and E. Strack, *Z. physiol. Chem.*, 1928, **177**, 177 (*Chem. Abs.*, 1928, **22**, 3891).
[23] W. C. Haynes, F. H. Stodola, J. M. Locke, T. G. Pridham, H. F. Conway, V. E. Sohns, and R. W. Jackson, *J. Bacteriol.*, 1956, **72**, 412.

dried and evaporated to dryness. The residue was chromatographed on a column (Kieselgel G–20% Celite 545). Phenazine-1-carboxylic acid was eluted with chloroform. It was recrystallized (ethanol–5% dimethylformamide); m.p. 241—243° (lit.,[16a] 238—240°); yield 150—170 mg l^{-1}. 2-Hydroxyphenazine-1-carboxylic acid was eluted with 1% acetic acid in chloroform. It was recrystallized from benzene–ethanol; yield 40—60 mg l^{-1}. [It gave methyl 2-methoxyphenazine-1-carboxylate, m.p. 127—129° (lit.,[16c] 129°) with diazomethane.] Finally 2-hydroxyphenazine was eluted with chloroform–1% acetic acid–5% methanol. It was recrystallized (benzene–ethanol); m.p. 247—249° (lit.,[24] 253—254°); yield 15—20 mg l^{-1}.

Administration of Deuteriated Compounds.—The compounds were administered to the cultures (*Ps. aeruginosa*: 1 l of culture; *Ps. aureofaciens*: 600—800 ml of culture) in aqueous solution, sterilized by filtration, in four or five evenly spaced batches over a period of growth (*ca.* 50 h) beginning when pigment production became apparent (*ca.* 20 h after innoculation of the growth medium). The pigments were isolated 24 h after the last feed unless otherwise stated.

It was convenient to dissolve 6,7,8,9-tetradeuterio-1-carboxy-5-methylphenazinium chloride in conc. hydrochloric acid (5 mg ml^{-1}) and then to neutralize the solution with aqueous potassium carbonate; final concentration 0·2 mg ml^{-1}. 1,2,3,4-Tetradeuterio-5-methylphenazinium methosulphate dissolved readily in water. Both phenazinium salts were unstable in solution and so solutions were only prepared just prior to use. 6,7,8,9-Tetradeuteriophenazine-1-carboxylic acid, 2,4-dideuteriophenazine-1,6-dicarboxylic acid, and the deuteriated 1-hydroxyphenazine were dissolved in aqueous sodium hydroxide (0·1N; *ca.* 20 mg ml^{-1}). The solution was neutralized with 2N-hydrochloric acid and diluted; final concentration *ca.* 0·2 mg ml^{-1}.

Determination of Deuterium Enrichment of the Metabolites.—The determination was carried out by mass spectrometry at low resolution; constant sample pressure was maintained. Most of the samples showed deuterium enrichment of *ca.* 15% or less and the following precautions were observed. A major fragment ion of high mass as well as the molecular ion was used for estimating the deuterium content, to avoid including the contribution of a chance impurity. The high mass fragment ion was selected with adjacent peaks of low intensity, and as the materials used contained a minimum of two, and usually three or four deuterium atoms, a particular deuteriated species gave essentially a single peak and the contribution from undeuteriated material of ions in the cluster around its corresponding peak made only a small contribution to the peak from deuteriated material. These contributions, where significant, were allowed for by running a spectrum of a non-deuteriated sample at the same time. All enrichments in the Tables are average values from several determinations.

For mass spectral analysis phenazine-1-carboxylic acid was converted into its methyl ester (diazomethane), 2-hydroxyphenazine-1-carboxylic acid was converted into methyl 2-methoxyphenazine-1-carboxylate (diazomethane), and pyocyanin was dequaternized to give 1-hydroxyphenazine. The following ions, given for non-deuteriated species, were used for the estimation: 1-hydroxyphenazine and 2-hydroxyphenazine: *m/e* 196 (M^+) and 168 ($M^+ - CO$); methyl phenazine-1-carboxylate: *m/e* 238 (M^+) and 207 ($M^+ - CH_3O$); methyl 2-methoxyphenazine-1-carboxylate: *m/e* 268 (M^+) and 237 ($M^+ - CH_3O$).

Administration of 2,4-Dideuteriophenazine-1,6-dicarboxylic Acid to Ps. aeruginosa.—In two experiments 2,4-dideuteriophenazine-1,6-dicarboxylic acid (23·0 and 24·0 mg) was administered to *Ps. aeruginosa*. The deuteriated acid was also administered over the period from 7 to 16 h after innoculation of the growth medium and the pyocyanin was isolated on this occasion after a further 55 h. None of the samples of 1-hydroxyphenazine derived from the three samples of pyocyanin (27·2, 50·8, and 59·6 mg) showed any incorporation of phenazine-1,6-dicarboxylic acid.

[1/1763 *Received, September 24th,* 1971]

[24] F. Kehrmann and F. Cherpillod, *Helv. Chim. Acta*, 1924, **7**, 973.