Incorporation of [^{14}C]Shikimate into Phenazines and their Further Metabolism by *Pseudomonas phenazinium*

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1. During growth of *Pseudomonas phenazinium* on L-threonine medium, phenazine pigment formation commenced early and 1,6-dihydroxyphenazine 5,10-dioxide (iodinin) was the major component. Growth on L-[U- ^{14}C]threonine showed that when growth was complete about 25% of the label had been incorporated into phenazines and 30% into cell substance. 2. The addition of D-[2,3,4,5(n)- ^{14}C]shikimate to cultures at different phases of growth showed that the greatest efficiency of incorporation (about 70%) occurred in the mid- to late-exponential phase. Phenazines accounting for most of the ^{14}C supplied were iodinin and 9-hydroxyphenazine-1-carboxylate plus 2,9-dihydroxyphenazine-1-carboxylate. Radioactivity incorporated into cell substance was about one-third of the amount found in phenazines. 3. Kinetic studies showed that radioactivity from a pulse of [^{14}C]shikimate was incorporated into phenazines immediately, without a discernible lag, and into all detectable phenazines simultaneously rather than sequentially. 4. Radioactive phenazines isolated from culture media were fed to growing cultures and their metabolism was studied. The results supported a scheme for the biosynthesis of iodinin and 1,8-dihydroxyphenazine 10-monoxide by a branched pathway. 5. It is proposed that phenazine-1,6-dicarboxylate is the common precursor of all naturally occurring phenazines.

Although shikimate is known to be a precursor of many phenazines (see Ingram & Blackwood, 1970), and chorismate has been shown to be the branch-point compound leading to pyocyanine (Calhoun *et al.*, 1972; Longley *et al.*, 1972), little is known of the route from chorismate to individual phenazines. Information on the assembly of the first-formed phenazine from a derivative of shikimate has been obtained from the elegant studies by Hollstein & McCamey (1973) and Herbert *et al.* (1974, 1976) of iodinin biosynthesis. These have shown that a diagonally symmetrical pairing of shikimate derivatives takes place. Interest now centres on details of the pathway leading from chorismate to the first-formed phenazine and the routes leading from this still unidentified common precursor to individual phenazines.

We have reported previously that *Pseudomonas phenazinium* produces ten phenazine pigments, predominantly iodinin, in particularly good yield when grown on L-threonine medium (Bell & Turner, 1973; Byng & Turner, 1975). On the basis of altered patterns of phenazine production and cross-feeding experiments with pigmentation mutants of the pseudomonad, we have proposed a scheme for the metabolic interrelationship of all ten phenazines (Byng & Turner, 1976). We describe here the formation of radioactive phenazines from [^{14}C]shikimate and their further metabolism by *Pseudomonas phenazinium*.

Materials and Methods

Micro-organisms and media

Pseudomonas phenazinium (N.C.I.B. 11027) was grown in liquid culture on basal-salts medium supplemented with L-threonine as the sole source of carbon and nitrogen (Bell & Turner, 1973). The concentration of L-threonine used was 2 g/litre and the pH of the medium was 5.5. Pigmentation mutants of the pseudomonad, with patterns of phenazine production characteristically different from that of the wild-type, were also grown on L-threonine + salts medium (Byng & Turner, 1976). Cultures of all strains were maintained on nutrient agar by standard procedures. Growth in liquid cultures was followed by measuring the $A_{600}^{1\text{cm}}$. At 600 nm the presence of phenazine pigments did not interfere with measurements of bacterial cell density.

Extraction, separation and identification of phenazines

Phenazines were extracted from acidified cultures by shaking with chloroform and separated by t.l.c. by the modified procedures of Gerber & Lechevalier (1964) as described previously (Byng & Turner, 1975, 1976). In some cases cultures were initially centrifuged at 12000g for 30 min to remove bacteria and particles of iodinin. Phenazines in the supernatant, and pellet material resuspended in dilute HCl, were then extracted separately. The concentration of chloroform extracts before chromatography was

limited by the poor solubility of some phenazines, particularly 1,8-dihydroxyphenazine 10-monoxide, and any precipitated material was redissolved for quantitative work. The routine fractionation of phenazines by t.l.c. on silica gel G yielded up to seven pigment bands (Byng & Turner, 1975) but did not separate 1,6-dihydroxyphenazine, its 10-monoxide and 5,10-dioxide (iodinin) (band 1) or 9-hydroxyphenazine-1-carboxylate and 2,9-dihydroxyphenazine-1-carboxylate (band 7). 'Band 1' compounds were readily separated by t.l.c. on cellulose acetate in the solvent described by Byng & Turner (1975). 'Band 7' substances were not separable by any t.l.c. procedure tested. Their methyl derivatives, obtained by treatment with diazomethane, could be separated by t.l.c. on silica gel G, but this was not done as a routine.

Phenazines separated by chromatography were identified as a routine by their colour, R_F values and spectral characteristics after elution with the appropriate solvent (Byng & Turner, 1975). One of the ten phenazines characterized but originally of unknown identity was later shown to be phenazine-1,6-dicarboxylate (Byng & Turner, 1976).

Radioactive phenazines were located on t.l.c. plates by using a Panax automatic scanner.

Spectrophotometric assay of phenazines

Chloroform extracts of acidified bacterial cultures were clarified by centrifuging at 1000g for 5 min, appropriately diluted with chloroform, and spectra were obtained by using a Unicam SP.800 scanning spectrophotometer. An estimate of the total phenazine content was obtained from the absorption at 270–280 nm. After separation by t.l.c., pigment bands were scraped off, phenazines eluted with the appropriate solvent, diluted and spectra recorded. The concentrations of individual phenazines were assayed by using extinction coefficients determined with the purified materials. The solvent, $\lambda_{\max}$, and ϵ values used were: 1,6-dihydroxyphenazine, chloroform, 272 nm, 5300; 1,6-dihydroxyphenazine 5,10-dioxide, chloroform, 290 nm, 5400; phenazine-1-carboxylate, chloroform, 252 nm, 6000; and 1,8-dihydroxyphenazine 10-monoxide, ethanol, 283 nm, 4300.

Radio-tracer method for measuring phenazine production

Cultures of *Ps. phenazinium* were grown on standard L-threonine+salts medium containing 2 g (150 μ Ci) of L-[U- 14 C]threonine. When growth was complete, cultures were extracted with chloroform, and phenazines separated as described above. The radioactivity of individual phenazines was measured by liquid-scintillation counting. Phenazines were eluted from t.l.c. adsorbents with the appropriate solvent, and samples were transferred to scintillation vials

and dried under N_2 . Water (1 ml) was then added, followed by 10 ml of Aquasol scintillation 'cocktail' (NEN Chemicals G.m.b.H., Dreieichenhain, W. Germany). Alternatively samples on silica gel or cellulose acetate powder, removed from t.l.c. plates, were added to vials and suspended in a gel formed by adding 2 ml of water and 10 ml of Aquasol. Samples were counted for radioactivity in the combined ^{14}C - 3H channel, in an Intertechnique SL 30 liquid-scintillation spectrometer, and counts converted into d.p.m. by the external-standard-ratio method based on carbon tetrachloride quenching. Highly coloured samples and samples containing silica or cellulose powder were corrected for by adding an internal standard of [1- ^{14}C]hexadecane dissolved in Aquasol. Efficiencies of 70–75% were observed as a routine.

Preparation of radioactive phenazines from [^{14}C]shikimate

To cultures of *Ps. phenazinium* growing exponentially on L-threonine+salts medium ($A_{600}^{1.6}$) was added 0.5 μ Ci (10 nmol) of D-[2,3,4,5(n)- ^{14}C]shikimate/15 ml. When growth was complete, radioactive phenazines were extracted, separated and assayed as described above. Radioactive 1,6-dihydroxyphenazine was isolated from cultures of *Ps. phenazinium* strain D34 (Byng & Turner, 1976).

Measurement of radioactivity assimilated by bacteria

Culture samples were filtered through Millipore bacterial filters (HAWP, 0.45 μ m pore size), which were then washed with fresh medium. Filters with retained bacteria were dried, attached to planchets and their radioactivity was measured with an end-window counter by standard procedures. If necessary, particles of iodinin were removed from culture medium by pre-filtration through a funnel with sintered glass disc, porosity 3 (Baird and Tatlock, Chadwell Heath, Essex, U.K.).

Chemicals

Radioactive L-[U- ^{14}C]threonine and D-[2,3,4,5(n)- ^{14}C]shikimate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Silica gel was obtained as Kieselgel G from E. Merck A.G., Darmstadt, Germany, and cellulose acetate (40% acetylated) from Machery Nagel and Co., Düren, Germany. L-Threonine was obtained from SAS Scientific Chemicals, London WC1B 4DR. Other chemicals obtained commercially were from BDH, Poole, Dorset, U.K., and were A.R. grade or of the highest quality available. Samples of phenazine-1-carboxylate, 6-hydroxyphenazine-1-carboxylate, 9-hydroxyphenazine-1-carboxylate and phenazine-1,6-dicarboxylate were generous gifts from Professor F. G. Holliman and Dr. R. B. Herbert.

Table 1. *Phenazine biosynthesis by Pseudomonas phenazinium* (wild-type)

The bacteria were grown on L-[U- 14 C]threonine (0.2g, 15 μ Ci/100ml) as the sole source of carbon and nitrogen as described in the Materials and Methods section. When growth had ceased, phenazines were extracted from the medium, separated and assayed by spectrophotometric and radio-tracer methods (see the Materials and Methods section). 1,6-Dihydroxyphenazine and 6-hydroxyphenazine-1-carboxylate were found in only trace amounts (see the text). No other phenazines were detected. Abbreviation: n.d., not done.

Phenazine	Radioactivity from L-[U- 14 C]threonine (% recovery)	Amount (μ mol/l)
1,6-Dihydroxyphenazine 5,10-dioxide (iodinin)	16.0	1100
'Band 7' material*	6.0	n.d.
Phenazine-1-carboxylate	0.8	47
1,8-Dihydroxyphenazine 10-monoxide	0.3	34
Phenazine-1,6-dicarboxylate	0.2	n.d.

* 9-Hydroxyphenazine-1-carboxylate plus 2,9-dihydroxyphenazine-1-carboxylate.

Results

Pigment formation during growth of *Pseudomonas phenazinium*

Preliminary experiments showed that pigment formation commenced early during growth on L-threonine medium but that only small amounts of iodinin accumulated before growth was about half complete ($A_{600} = 1.0$). When cultures in the stationary phase were extracted, i.e. after pigment formation had stopped, iodinin was found to be the major component, with minor amounts of four or five other phenazines present. These results were obtained by spectrophotometric assays. The relative amounts of the different phenazines produced, and the amount of growth substrate used for bacterial growth, were conveniently measured by a radio-tracer method (see the Materials and Methods section). The pseudomonad was inoculated into L-[U- 14 C]threonine+salts medium, allowed to grow to completion and the pigments were extracted and fractionated as described. The results of spectrophotometric and radio-tracer experiments agreed well and suggested that about 25% of the L-threonine supplied as growth substrate was incorporated into phenazines (Table 1). The radio-tracer experiment showed that a further 30% of the 14 C supplied as L-threonine was incorporated into the bacteria. The remaining radioactivity was lost, probably as $^{14}\text{CO}_2$.

When cultures were extracted at different phases of growth, some differences in the pattern of phenazine production were found. 6-Hydroxyphenazine-1-carboxylate accumulated transiently during the mid-exponential phase, whereas 1,8-dihydroxyphenazine and its 10-monoxide were not detectable until the late-stationary phase.

Phenazine biosynthesis from [14 C]shikimate

When [14 C]shikimate was added to cultures of *Ps. phenazinium* at different stages of growth, and these were allowed to grow fully, good incorporation of

Fig. 1. Effect of growth phase on the incorporation of [14 C]shikimate into phenazines by *Pseudomonas phenazinium*

Radioactive shikimate (0.5 μ Ci, 10nmol) was added to cultures of the pseudomonad (15ml), growing on L-threonine+salts medium, at different times after inoculation. The cell density (○) was measured in each case. When cultures had reached the stationary phase they were acidified, extracted with chloroform, and the radioactivity of the extract (●) was measured as described in the Materials and Methods section.

label into total phenazines occurred after the exponential and early-stationary phases. The most efficient incorporation (65–75%) occurred when [14 C]shikimate was added towards the end of the exponential growth phase ($A_{600} = 0.8$ –1.9) as indicated in Fig. 1. When the radioactivity of individual phenazines was analysed, the predominant phenazines

were iodinin (45%), 'band 7' material, i.e. 9-hydroxyphenazine-1-carboxylate plus 2,9-dihydroxyphenazine-1-carboxylate (30%), phenazine-1-carboxylate (15%), 1,8-dihydroxyphenazine 10-monoxide (8%) and phenazine-1,6-dicarboxylate (2%) in the relative amounts shown. The relative radioactivities of phenazines recovered after growth was complete did not vary significantly with the time of addition of [^{14}C]shikimate.

Kinetic experiments showed that, when a 10 nmol pulse of [^{14}C]shikimate was added to 15 ml cultures of *Ps. phenazinium* during exponential growth ($A_{600} = 1.0$), most of the label appeared in phenazines or other products within about 30 min. Radioactivity was incorporated immediately, without a discernible lag, and preferentially into phenazines. Of the total radioactivity recovered, 75% was found in the phenazine extract and 25% in the bacteria themselves. When samples were removed at short time-intervals and the phenazine fraction was analysed, it was found that radioactivity from added [^{14}C]shikimate appeared to enter all detectable phenazines simultaneously rather than sequentially (Fig. 2). In separate experiments where the radioactivity entering different phenazines was followed over a longer time-period, it was confirmed that [^{14}C]shikimate was rapidly incorporated and that their relative radioactivities did not change markedly with time (Table 2). Radioactive 1,8-dihydroxyphenazine 10-monoxide was formed, in only small amounts, about 15 h after the addition of [^{14}C]shikimate.

Further metabolism of radioactive phenazines

Radioactive phenazines were produced biosynthetically from [^{14}C]shikimate and isolated as described above (see also the Materials and Methods section). Each phenazine was dissolved in ethanol and a small volume added to cultures of *Ps. phenazinium* growing in the mid- and late-exponential phases of growth. Wild-type and mutant strains of the pseudomonad were used, the probable positions

Fig. 2. Time-course of phenazine synthesis from [^{14}C]shikimate

Radioactive shikimate was added to cultures of *Ps. phenazinium* ($A_{600} = 1.0$) under the conditions described for Fig. 1. Cultures were acidified by the addition of conc. HCl at the times shown, extracted with chloroform and radioactive phenazines separated and counted for radioactivity as described in the Materials and Methods section. The radioactivity of individual phenazines is expressed as the percentage recovered from the [^{14}C]shikimate added. The [^{14}C]phenazines found were iodinin (●), 'band 7' material (○), phenazine-1-carboxylate (▲), phenazine-1,6-dicarboxylate (△) and 6-hydroxyphenazine-1-carboxylate (□).

of the metabolic lesions in the mutants (Byng & Turner, 1976) being indicated in Scheme 1. After the growth of cultures was complete, phenazines were

Table 2. Incorporation of [^{14}C]shikimate into individual phenazines by *Pseudomonas phenazinium* (wild-type) D-[2,3,4,5(n)- ^{14}C]Shikimate (0.5 μCi , 10 nmol) was added to cultures of *Ps. phenazinium* (15 ml) growing on L-threonine+ salts medium (see the Materials and Methods section) at $A_{600} = 1.0$. At the time intervals indicated, cultures were removed and the radioactivity of individual phenazines was measured as described in the Materials and Methods section. A small trace of 6-hydroxyphenazine-1-carboxylate was found in only the 1 h sample.

Phenazine	Sampling time (h)	Radioactivity recovered (%)			
		1	3	16	42
1,6-Dihydroxyphenazine-5,10-dioxide (iodinin)	...	16.5	18.5	19.5	20.5
'Band 7' material*	...	18.5	19.5	12.0	11.0
Phenazine-1-carboxylate	...	12.0	13.0	10.0	10.0
1,8-Dihydroxyphenazine-10-monoxide	...	0	0	1.5	3.0
Phenazine-1,6-dicarboxylate	...	3.0	3.0	3.0	3.0

* 9-Hydroxyphenazine-1-carboxylate plus 2,9-dihydroxyphenazine-1-carboxylate.

Table 3. *Metabolism of radioactive phenazines by growing cultures*

Radioactive phenazines, prepared biosynthetically from [^{14}C]shikimate, were isolated from cultures of *Ps. phenazinium* and purified as described in the Materials and Methods section. Solutions in ethanol or dimethyl sulphoxide (0.5 ml, 0.05 μCi) were added to growing cultures of the pseudomonad (15 ml) in the mid- and late-exponential phases of growth. When growth was complete ($A_{600} = 2.0$), cultures were extracted, phenazines separated and their radioactivities measured (see the Materials and Methods section). Results are expressed as the radioactivity recovered from the labelled precursor fed to the bacterial strains shown at $A_{600} = 1.0$. Values in parentheses were obtained when the precursor was added at $A_{600} = 1.5$. Radioactivity was found only in the products indicated. The recovery of radioactivity in products and unused precursor was about 75%. Abbreviation: n.d., not done.

Precursor	Product	Radioactivity recovered (%) from cultures of the strains shown			
		Wild-type	F8	9Z	18Z
1,6-Dihydroxyphenazine	1,6-Dihydroxyphenazine 5-monoxide	13 (14)	—	6 (n.d.)	8 (n.d.)
	Iodinin	55 (80)	—	69 (n.d.)	73 (n.d.)
Phenazine-1-carboxylate	'Band 7' material*	19 (23)	15 (13)	44 (54)	62 (52)
	1,8-Dihydroxyphenazine 10-monoxide	2 (1)	3 (3)	5 (6)	7 (3)
	Iodinin	2 (2)	0 (0)	2 (2)	2 (1)
'Band 7' material*	1,8-Dihydroxyphenazine 10-monoxide	6 (n.d.)	11 (7)	6 (5)	7 (4)

* 9-Hydroxyphenazine-1-carboxylate plus 2,9-dihydroxyphenazine-1-carboxylate

isolated and their radioactivity was measured by the standard procedures.

When 1,6-dihydroxy[^{14}C]phenazine was fed to cultures of wild-type and mutant strains, up to 80% of the added label was recovered in iodinin. The only other radioactive product detected was 1,6-dihydroxyphenazine 5-monoxide (see Table 3). No radioactivity was found in 'band 7' material.

[^{14}C]Iodinin did not give rise to any labelled product when added to growing cultures (as a solution in dimethyl sulphoxide).

The addition of [^{14}C]phenazine-1-carboxylate to cultures yielded 'band 7' material as the major labelled product, with some radioactive 1,8-dihydroxyphenazine 10-monoxide. The extent of conversion by the non-pigmented mutants was greater than that by the pigment-forming strains (see Table 3). Additional experiments with the wild-type showed that up to 42% of [^{14}C]phenazine-1-carboxylate was metabolized to 'band 7' material after addition in the mid-exponential phase of growth, but that the efficiency of conversion declined rapidly thereafter.

When radioactive 'band 7' material was fed back to cultures, only 1,8-dihydroxyphenazine 10-monoxide became labelled (Table 3). Additional experiments again showed that the time of addition of the radioactive precursor was important. The highest incorporation occurred when the growth of the wild-type was virtually complete. In one experiment 18, 19 and 20% conversion of 'band 7' material into the 1,8-diol 10-monoxide occurred when the additions were made at $A_{600} = 1.0$, 1.5 and 2.0 respectively.

Discussion

One of the most notable findings reported here was that, during the growth of *Ps. phenazinium* on L-

threonine, almost half of the carbon used for biosynthetic purposes was channelled into phenazine formation. Similarly, of the [^{14}C]shikimate assimilated during exponential growth, only 25% was incorporated into bacterial substance, compared with 75% incorporated into phenazines. The mechanism whereby, under certain circumstances, the intermediary metabolism of *Ps. phenazinium* is directed to the biosynthesis of these secondary metabolites is of considerable interest.

The results of the radio-tracer experiments reported here support the operation of the route proposed for phenazine biosynthesis in *Ps. phenazinium* (Scheme 1). The metabolism of radioactive phenazines by mutant strains 9Z and 18Z showed that, although non-pigmented, they possessed the enzymes needed to form 1,8-dihydroxyphenazine 10-monoxide, as well as iodinin (Byng & Turner, 1976), from precursor phenazines. Although the evidence for phenazine-1,6-dicarboxylate acting as a branch-point compound is indirect, based on its accumulation by some pigmentation mutants (Byng & Turner, 1976), experiments with other biosynthetically generated [^{14}C]phenazines suggest that iodinin and 1,8-dihydroxyphenazine 10-monoxide are formed by different pathways. Radioactivity from 1,6-dihydroxyphenazine fed to three strains of *Ps. phenazinium* was found in its 10-monoxide and 5,10-dioxide (iodinin), but in none of the proposed intermediates leading to 1,8-dihydroxyphenazine 10-monoxide. 6-Hydroxy[^{14}C]phenazine-1-carboxylate was not produced by *Ps. phenazinium* in amounts sufficient for testing, but its metabolism to iodinin by *Brevibacterium iodinin* has been described (Herbert *et al.*, 1972). Radioactivity from phenazine-1-carboxylate, in contrast, was metabolized to inter-

Scheme 1. Scheme for phenazine biosynthesis in *Pseudomonas phenazinium*

Section 1 represents the route from chorismate to the common precursor of the phenazines found. Reactions 2 and 3 represent hydroxylative decarboxylation, reactions 4, 5 and 10 *N*-oxidation, reactions 6 and 9 direct decarboxylation and reactions 7 and 8 hydroxylation steps. The probable positions of the metabolic lesions in the mutant strains used are indicated.

mediates of the branch leading to 1,8-dihydroxyphenazine 10-monoxide. 'Band 7' material (9-hydroxyphenazine-1-carboxylate and 2,9-dihydroxyphenazine-1-carboxylate, which were not separable) gave 1,8-dihydroxyphenazine 10-monoxide only. Although this monoxide was not a major product, its late formation suggested that it was a slowly formed end product.

Hollstein *et al.* (1976) have shown that phenazine-1,6-dicarboxylate, supplied as its dimethyl ester to *Pseudomonas aureofaciens*, acts as a precursor of phenazine-1-carboxylate, thus lending support to step 6 proposed in Scheme 1. In turn, the mono-carboxylate is believed to act as a precursor of its betaine and pyocyanine (Flood *et al.*, 1970, 1972), 1-hydroxyphenazine and its 10-monoxide (Herbert *et al.*, 1972), aeruginosins A and B (Hansford *et al.*, 1972; Bentley & Holliman, 1970), 2-hydroxyphenazine and its 1-carboxylate (Flood *et al.*, 1972), and, as reported here, several phenazines metabolized to 1,8-dihydroxyphenazine 10-monoxide. From this evidence and a consideration of the structures of all naturally occurring phenazines, it seems likely that phenazine-1,6-dicarboxylate is their common precursor, capable of modification by a series of well-known metabolic reactions. Podojil & Gerber (1970) previously proposed 4,9-dihydroxyphenazine-1,6-dicarboxylate as the key intermediate, but this compound has never been found.

Little is known of the enzymology of phenazine metabolism. The only reactions known to be catalysed by disrupted cells are the *N*-oxidation of 1,6-dihydroxyphenazine (Gerber & Lechevalier, 1965) and 1-hydroxyphenazine (Gerber, 1966) by sonicated suspensions of *Brevibacterium iodinium*. Although the availability of [¹⁴C]phenazines may aid such studies, the poor solubility of phenazines in aqueous solvents is a limiting factor, and alternative approaches remain to be exploited. Phenazine mutants D34 and E10 of *Ps. phenazinium* produce little or no phenazine *N*-oxides, suggesting that the same enzyme is responsible for the oxidation of both 1,6- and 1,8-dihydroxyphenazine (Byng & Turner, 1976). Similar considerations suggest that the sequential hydroxylative-decarboxylation reactions converting phenazine-1,6-dicarboxylate into 1,6-dihydroxyphenazine are catalysed by the same enzyme system. More detailed genetic studies may be expected to give information on the precise number of loci concerned in phenazine biosynthesis.

Details of the pathway leading from chorismate to

phenazines are still lacking. Potential precursors may be conveniently tested by feeding to phenazine-less mutants blocked in this region, although preliminary cross-feeding experiments suggest that *Ps. phenazinium* may be impermeable to phenazine precursors (Byng & Turner, 1976).

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