

Further Observations on the Source of Nitrogen in Phenazine Biosynthesis

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It is concluded from experiments with (S)-[CO¹⁵NH₂]glutamine and [¹⁵N]ammonium sulphate that the biosynthesis of iodinin (1), in *Brevibacterium iodinum*, and of phenazine-1-carboxylic acid (7), in *Pseudomonas aureofaciens*, is primarily from a single nitrogen source (glutamine) and that the phenazines are constituted by combination of two units of the same precursor, as 5. Some observations are made on the inhibition and triggering of iodinin biosynthesis.

We have shown recently [1] that the amide nitrogen of glutamine is the immediate source of the nitrogen atoms in the phenazine, iodinin 1, and in 2-aminophenoxazinone (2), produced by cultures of *Brevibacterium iodinum*. We concluded that the biosynthesis of 1 and 2 is closely related and that these metabolites are derived from shikimic acid 3 via chorismic acid 4 and a hypothetical aminated intermediate 5, which could also be involved in anthranilic acid biosynthesis [2].

In experiments with (S)-[CO¹⁵NH₂]glutamine, the iodinin (1) formed, consistently showed a higher than statistical amount of dilabel (11.7% ¹⁵N₁, 5.0% ¹⁵N₂; 10.8 excess atom %) [1]. In contrast, [¹⁵N]ammonium sulphate gave rise to a very low abundance of dilabelled species (11.0% ¹⁵N₁, 0.3% ¹⁵N₂; 5.8 excess atom %). Two possible explanations were advanced for these observations, the first of which depends on the fact that precursors were administered in batches over several hours during iodinin production: A, the [¹⁵N]glutamine, but not [¹⁵N]ammonium sulphate (which is less efficiently utilized), for a time after administration of precursor, swamps the nitrogen pool used as a single primary source for both nitrogen atoms in phenazine biosynthesis; B, there are two immediate, and separate, sources for the two phenazine nitrogens both of which are labelled by [¹⁵N]glutamine but only one of which is effectively labelled by [¹⁵N]ammonium sulphate. We report here results which support the first explanation and refute the second.

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Two sets of experiments were carried out, both beginning at the onset of iodinin production. In the first, a solution of (S)-[CO¹⁵NH₂]glutamine was administered at a constant and low rate to *B. iodinum* cultures over 5.5 h. The iodinin isolated after a further 9.5 h showed good ¹⁵N-enrichment and the labelling conformed to a statistical distribution between ¹⁵N₁ and ¹⁵N₂ (Table I). In the second set, (S)-[CO¹⁵NH₂]glutamine and [¹⁵N]ammonium sulphate were each administered in a single pulse and the labelling in the iodinin was monitored at intervals thereafter. The results (Table II) show a high (non-statistical) ¹⁵N₂/¹⁵N₁ ratio at first for the iodinin formed from the glutamine which fell with time to approach a statistical ratio. Indeed the amount of ¹⁵N₂-species remained roughly constant with time which means that most of the dilabelled material was formed very shortly after administration of the precursor. On the other hand, the ¹⁵N₂/¹⁵N₁ ratio in the iodinin formed from [¹⁵N]ammonium sulphate remained statistical throughout the experiment. The total amount of label incorporated actually rose with time which means that label from this precursor was only very slowly made available for phenazine biosynthesis. These two sets of results are consistent only with explanation A

Table I. Administration of (S)-[CO¹⁵NH₂]glutamine to *B. iodinum* at a constant rate over 5.5 h; incorporation in iodinin 1.

Experiment	Excess atom [%]	¹⁵ N ₁ [%]	¹⁵ N ₂ [%]
1	7.3	12.9	0.8
2	9.6	15.1	2.0



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Table II. Incorporation of [^{15}N]-labelled precursors into iodinin **1**; precursors fed in one batch.

Iodinin isolated		[^{15}N]glutamine			[$^{15}\text{NH}_4$] SO_4		
		ϵ_{max}^*	$^{15}\text{N}_1$ [%]	$^{15}\text{N}_2$ [%]	ϵ_{max}^*	$^{15}\text{N}_1$ [%]	$^{15}\text{N}_2$ [%]
after	2.5 h	0.10	23.2	22.3	0.08	10.2	0.4
	3.5 h	0.18	16.2	11.7	0.12	11.9	0.9
	6.5 h	0.36	15.9	7.6	0.27	15.3	1.1

* Measured at 520 nm in CHCl_3 .

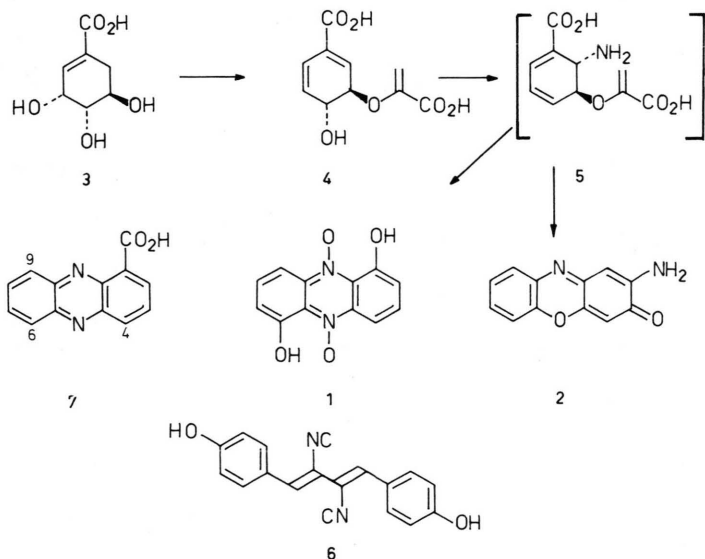
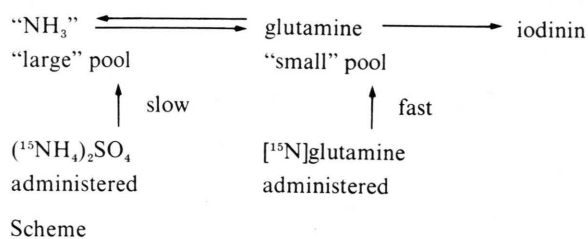
above. It follows that phenazine biosynthesis is by combination of two units of the same precursor, as **5**, formed by utilization of a single primary nitrogen source.

It is clear from these and earlier results [1] that (S)-[CO $^{15}\text{NH}_2$]glutamine is rapidly transported to the aminating enzyme(s) involved in phenazine biosynthesis swamping the pool used for a short time thus generating dilabelled iodinin, but also that other enzyme(s) quickly remove the excess glutamine label to another pool because even after 2.5 h a large amount of unlabelled iodinin had been formed. The conclusions are summarized in the Scheme.

The previous observations [1] that unlabelled ammonium sulphate did not affect (S) [CO $^{15}\text{NH}_2$]glutamine incorporation and unlabelled glutamine only slightly affected the [^{15}N]ammonium sulphate incorporation is consistent with this Scheme, in particular

the obviously very rapid metabolism of the glutamine fed.

Xanthocillin (**6**) is like the phenazines (as **1**) in being a symmetrical molecule containing two nitrogen atoms. Xanthocillin (**6**) has been shown to derive from two molecules of tyrosine [3]. Because, particularly, [^{15}N]tyrosine (fed in a single pulse) gave a low overall incorporation of ^{15}N relative to ^{14}C but a higher than statistical amount of [$^{15}\text{N}_2$]xanthocillin (**6**), it was concluded that the two "halves" of this



metabolite are formed by different pathways from tyrosine [*cf.* explanation B above]. In the light of our more extensive results for iodinin biosynthesis the results obtained for xanthocillin (**6**) may be reinterpreted in a way similar to those for iodinin (**1**) above. Further experiments to test this for xanthocillin are in hand.

In a final test of the possibility that microbial phenazines are formed from two different nitrogen-containing "halves", or different nitrogen sources, we have examined the incorporation of [^{15}N]ammonium sulphate, in the presence of unlabelled glutamine, into phenazine-1-carboxylic acid (**7**), produced by *Pseudomonas aureofaciens* [4]. If explanation B was correct labelling of only one of the two nitrogen atoms in **7** should have been observed in this experiment*. In this unsymmetrical phenazine, the

* It should be noted that the forthcoming results would only have been truly significant if unequal satellite intensities had been observed because biosynthesis by way of a symmetrical intermediate such as phenazine-1,6-dicarboxylic acid [6], if at any stage not enzyme-bound, would inevitably have led to symmetrization of any ^{15}N -label between the two nitrogen atoms.

^{13}C NMR signals for the carbon atoms flanking the two nitrogens (C-1 and C-9, C-4 and C-6) have different chemical shifts [5]; ^{15}N -enriched material would give rise to clearly distinguishable satellite signals due to ^{15}N – ^{13}C couplings for the carbon atoms two bonds away from the enriched nitrogen(s) [5]. The ^{13}C NMR spectrum of the methyl ester of the phenazine-1-carboxylic acid (**7**) obtained from the biosynthetic experiment showed ^{13}C – ^{15}N satellites for C-4, C-6 and C-9 (the signal for C-1 was too weak for observation) which were of equal relative intensity (Figure). These results are consistent with those obtained with iodinin above and provide no support for biosynthesis from two different nitrogen-containing molecules.

It is a notable feature of phenazine biosynthesis that the production of, *e.g.* iodinin (**1**) in *B. iodinum* begins quite suddenly in the late log phase growth of the cultures. We were interested to determine what factor(s) are responsible for triggering phenazine production. So far we have found that for *B. iodinum* the addition of neither phosphate nor EDTA (to check the influence of bivalent metal ions)

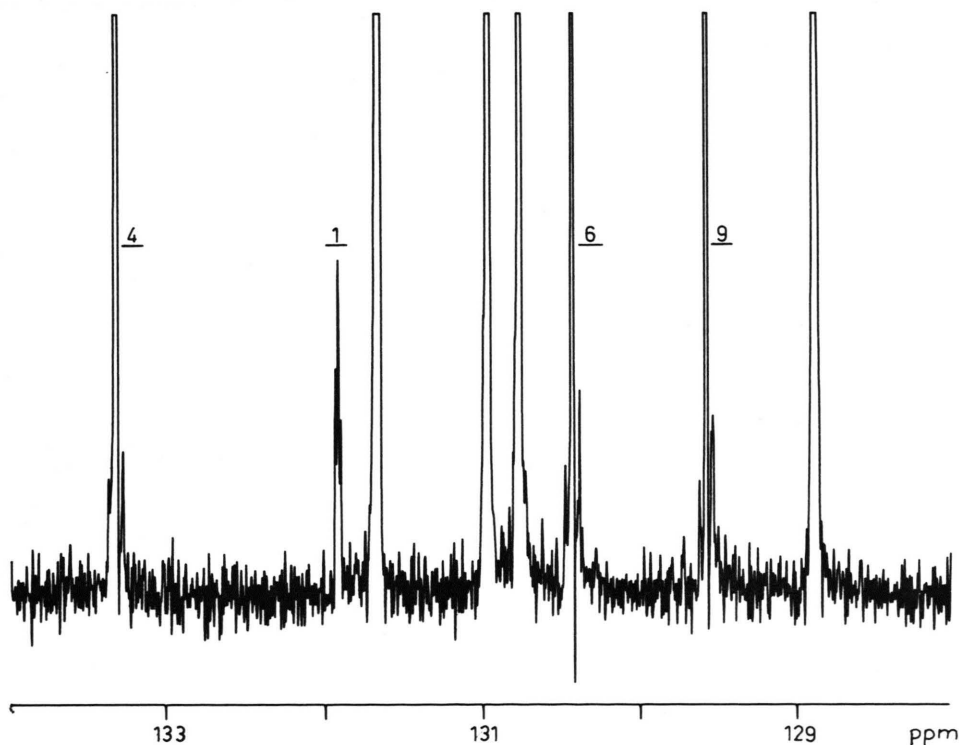


Fig. 1. Part of the 100 MHz ^{13}C -NMR spectrum of the naturally ^{15}N -enriched 1-carbomethoxyphenazine (**7**) (CDCl_3 ; TMS; 1956 scans; 4.0 sec acquisition time). The satellites next to the signals of C-4, C-6, and C-9 are due to the ^{15}N – ^{13}C couplings. The splitting of the signal of C-1 is caused by a slight temperature shift during the measurement.

showed any effect. Further, we could find no evidence for cell interactions dependent on cell concentration in dilution experiments. We found however that iodinin production was prevented by quite low concentrations of iodoacetamide [7] and diazooxonorleucine (DON) [8]. The latter compound is a structural analogue of glutamine [8, 9]. The former is an inhibitor for enzymes having thiol groups and notably for the glutamine binding enzyme (AS II) involved in anthranilic acid biosynthesis [10], and arguably [1] also in phenazine formation. Ammonia is able to substitute for glutamine in anthranilic acid synthesis [11] but addition of ammonium sulphate (or glutamine) along with an amount of iodoacetamide more than that required to completely prevent phenazine production led to a marginal restoration of iodinin biosynthesis. In the presence of this concentration of iodoacetamide label from both (S)-[CO¹⁵NH₂]glutamine and [¹⁵N]ammonium sulphate were incorporated into iodinin but at a very low level.

Experimental

General

Brevibacterium iodinum was cultured, and the iodinin isolated, as previously described [1, 12]. Compounds labelled with ¹⁵N (isotopic enrichment: 95%) were purchased from Rohstoffefuhr, Düsseldorf, FRG. Iodoacetamide and DON were purchased from Sigma London Chemical Company Ltd., England. Determination of ¹⁵N-label in iodinin was as described previously [1].

Continuous feeding of [CO¹⁵NH₂]glutamine to *B. iodinum*.

To each of two 100 ml cultures [¹⁵N]glutamine (11 mg) in water (5 ml) was administered continuously with a motor-driven syringe over a period of 5.5 h commencing at the onset of iodinin production (29 h after inoculation). 6.8 mg (A) and 6.9 mg (B) of iodinin were isolated after 44 h of incubation. (A): 7.3 excess atom %, 12.9% ¹⁵N₁, 0.8% ¹⁵N₂. (B): 9.6 excess atom %, 15.1% ¹⁵N₁, 2.0% ¹⁵N₂.

Feeding of [CO¹⁵NH₂]glutamine and (¹⁵NH₄)₂SO₄ in a single pulse to *B. iodinum*

Six 100 ml cultures were mixed and divided again into 6 portions 28 h after inoculation. To each of

three 100 ml cultures was added [¹⁵N]glutamine (13.7 mg) in water (2 ml). To each of the other three 100 ml cultures was added [¹⁵N]ammonium sulphate (19.3 mg) in water (2 ml). Iodinin was isolated after three different periods of time and the incorporation of nitrogen-15 was determined (Table II). The amount of iodinin produced was monitored by the UV absorption of the chloroform extract at 520 nm. The values were obtained after diluting the extract to a total volume of 1 l.

Addition of phosphate, EDTA, and water

At the beginning of the production of iodinin by 100 ml cultures of *B. iodinum* the addition per flask of up to 10 mg of Na₂HPO₃ in 2 ml of water or up to 120 mg of EDTA in 5 ml of water showed no effect on iodinin production (monitored by UV).

About one hour before the start of iodinin production (27 h of incubation) 100 ml cultures were each diluted with 200 ml of sterile water. There was no difference in the iodinin production of diluted and reference cultures.

Addition of iodoacetamide

The addition of iodoacetamide (2 mg) to a 100 ml culture of *B. iodinum* 28 h after inoculation completely prevented the synthesis of iodinin. The parallel addition of iodoacetamide (10 mg) and varying amounts of ammonium sulphate or glutamine (5, 50, and 100 mg) to 100 ml cultures, respectively, restored about 10% of the iodinin production. Feeding of iodoacetamide (10 mg) along with [¹⁵N]glutamine (17 mg) in two experiments and iodoacetamide (10 mg) together with [¹⁵N]ammonium sulphate (23 mg) in two further experiments led to the following incorporations ([¹⁵N]glutamine) 1.1% ¹⁵N₁, 1.6% ¹⁵N₂ and 1.0% ¹⁵N₁, 1.9% ¹⁵N₂; ([¹⁵N]ammonium sulphate) 0.4% ¹⁵N₁, 0% ¹⁵N₂ and 0.9% ¹⁵N₁, 0.2% ¹⁵N₂.

Addition of diazooxonorleucine (DON)

The addition of DON (0.2 mg) in water (1 ml) to a 100 ml culture of *B. iodinum* 27 h after inoculation completely prevented pigment production.

Administration of [¹⁵N]ammonium sulphate to *P. aureofaciens*

Pseudomonas aureofaciens INO4 [13] was cultured in two steps. Initiation culture: peptone 2%; glucose

1%; Speakman's solution A 0.5% and B 0.5%; pH 7.7 (NH_4OH). 20 ml cultures were incubated at 30 °C for 38 h. Production culture: (gl^{-1}) KH_2PO_4 5; Na-glucomate 10; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.5; Fe-III-citrate 0.01; $(\text{NH}_4)_2\text{SO}_4$ 5; pH 7.4 (KOH). 5 ml of initiation broth were transferred to 100 ml of production medium. The cultures were incubated at 30 °C. Phenazine production, which started after 4–5 h, was monitored by the UV absorption at 370 nm of chloroform extracts of acidified (pH 1) samples of the culture.

To each of the twelve 100 ml cultures [^{15}N]ammonium sulphate (22.4 mg) was administered after 5 h of incubation. After a further 0.5 h and then again after 2.5 h glutamine (17 mg) was added to each culture. 10 h after starting the production culture the combined broths were brought to pH 1 with hydrochloric acid and extracted thoroughly with the same volume of chloroform. The chloroform extract was dried (Na_2SO_4) and concentrated. The residual solution was methylated with ethereal

diazomethane overnight. Column chromatography on silica, using CHCl_3 /petrol ether 1:1 as eluent yielded first 1-carbomethoxyphenazine and then 1-carbomethoxy-2-methoxyphenazine. Further purification of these two metabolites was carried out with HPLC (Micropak Si 10; hexane/isopropanol 94:6; UV-detection at 370 nm). 1-Carbomethoxyphenazine: 60 mg; 6.7% $^{15}\text{N}_1$, 1.0% $^{15}\text{N}_2$; ^{13}C -NMR (100 MHz; CDCl_3 , TMS) 129.6 ppm (C-6), ^2J (N-C) 8.2 Hz, 130.5 ppm (C-9), ^2J (N-C) 8.4 Hz, 133.3 ppm (C-4), ^2J (N-C) 9.4 Hz. 1-Carbomethoxy-2-methoxyphenazine: 21 mg; 6.7% $^{15}\text{N}_1$, 1.1% $^{15}\text{N}_2$.

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