Biosynthesis of 1,6-Phenazinediol 5,10-Dioxide (Iodinin). Incorporation of Shikimic Acid*

Miloslav Podojil† and Nancy N. Gerber‡

ABSTRACT: A chemical degradation of the iodinin molecule was devised. With it, labeled iodinin isolated from a culture of *Brevibacterium iodinum*, after adding DL-[1,6-^{14}C]- or [*G*-^{14}C]shikimic acid, was degraded and the activity of the products determined. The incorporation patterns showed that two shikimic acid units were incorporated into the phenazine ring.

The relative incorporation of radioactivity from [*G*-^{14}C]-shikimic acid, [1- or 2-^{14}C]acetate, or [*G*-^{14}C]glutamic acid into iodinin and respiratory carbon dioxide showed that shikimic acid was not metabolized very efficiently; thus scrambling of the label was negligible.

Studies on the biosynthesis of phenazines have been carried out on pyocyanine (MacDonald, 1967; Chang and Blackwood, 1968; Ingledew and Campbell, 1969; Hollstein *et al.*, 1966), phenazine-1-carboxylic acid (Levitch, 1961; Levitch and Stadtman, 1964; Chang and Blackwood, 1968; Hollstein *et al.*, 1966), phenazine-1-carboxamide (= oxychlororaphine) (Takeda and Nakanishi, 1959; Chang and Blackwood, 1968; Carter and Richards, 1961), 2-hydroxyphenazine (Levitch and Reitz, 1966), and iodinin (Podojil and Gerber, 1967). The results have usually implicated shikimic acid as a precursor of the phenazine ring. However, no degradations of specifically labeled phenazines biosynthesized from shikimic acid have been carried out in order to determine the pattern of labeling.

The relatively good incorporation of shikimic acid into iodinin indicated that its synthesis is similar to those of the other phenazines studied (Podojil and Gerber, 1967). Therefore we decided to use specifically labeled shikimic acid and determine the radioactivity of suitable degradation products.

Materials and Methods

Brevibacterium iodinum no. 26 was obtained from Dr. R. E. Gordon of Rutgers University. The maintenance of the strain, the preparation of the inoculum, the fermentation conditions, and the isolation, purification, and assay of iodinin have all been described (Gerber and Lechevalier, 1964, 1965; Podojil and Gerber, 1967). DL-[1,6-^{14}C]Shikimic acid (specific activity 8.7 mCi/mmole) was supplied by Calbiochem. [*G*-^{14}C]Shikimic acid (specific activity 7.6 mCi/mmole) was purchased from New England Nuclear Corp. [1-^{14}C]Acetic acid (specific activity 28.2 mCi/mmole), [2-^{14}C]acetic acid (specific activity 21.8 mCi/mmole), and [*U*-^{14}C]glutamic acid (specific activity 11.5 mCi/mmole) were obtained from The Radiochemical Centre, Amersham. Labeled substrates were added to the 36-hr-old culture (10 μCi/flask) which was then reincubated for an additional 48 hr. Radioactive iodinin isolated from ten flasks (27 mg) was recrystallized from pyridine with carrier iodinin (190 mg) to constant activity (yield 205 mg). The respiratory carbon dioxide was collected by passing a stream of sterile carbon dioxide free air through the fermentation flask at 100 ml/min and then through a companion flask (250-ml erlenmeyer) containing 50 ml of 1 N sodium hydroxide.

1,6-Phenazinediol (**2**). Iodinin (200 mg) was dissolved in 200 ml of pyridine, then 120 mg of Zn dust and 2 ml of 10% HCl was added. The mixture was warmed and swirled a few moments until it became deep blue, then 5–10 min longer. It was poured into 200 ml of water, acidified to pH 4 with hydrochloric acid, and then extracted with $CHCl_3$. The $CHCl_3$ solution, washed several times with water, then evaporated to dryness, yielded 145 mg of 1,6-phenazinediol as brown crystals, identical (melting point and absorption spectrum) with an authentic specimen (Akabori and Nakamura, 1959).

Pyrazinetetracarboxylic Acid (**3**). To 2.5 ml of a 1% KOH solution was added 140 mg of 1,6-phenazinediol and, in portions, a solution of 1700 mg of potassium permanganate in 10 ml of water. After 2 hr on the steam bath the excess permanganate was decolorized with ethanol. The manganese dioxide was removed by suction filtration and washed twice. The combined filtrate and washings (85 ml) were passed through a 25-ml volume sulfonic acid ion-exchange resin column (IR-120-CP in the H^+ form). The combined eluate and washings (150 ml) were taken to dryness in the vacuum oven and the residue was recrystallized from 2.5 ml of 20% HCl to furnish 45 mg of shiny yellowish plates (mp 205–206°) with gas evolution: $\lambda_{max}^{H_2O}$ 295 mμ, $E_{1cm}^{1\%}$ 300. The mixture melting point with an authentic sample was not depressed (Light and Hauser, 1961).

Decarboxylation of Pyrazinetetracarboxylic Acid to Pyrazine (**4**) (*British Patent 560965*). Pyrazinetetracarboxylic acid (20 mg) was suspended in dibutyl phthalate (50 ml). The temperature of the mixture was raised to 190° and held

* From the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia, and the Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey. *Received April 2, 1970.* Supported by U. S. Public Health Service Grant AI-06230 and International Atomic Energy Agency, Vienna, Agency Research Contract No. 845/RB.

† Part of this work was performed during tenure of a Waksman–Merck postdoctoral fellowship at the Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, N. J.

‡ To whom to address correspondence.

TABLE I: Radioactivity of Degradation Products of Iodinin after Incorporation of DL-[1,6-^{14}C]- or [G-^{14}C]Shikimic Acid.

	DL-[1,6-^{14}C]Shikimic acid					[G-^{14}C]Shikimic acid			
			Rel %						
				Calcd					
Compound	dpm[a]	rma[b] × 10^{-3}	Found	1 mole of Shikimic Acid	2 moles of Shikimic Acid	dpm[a]	rma[b] × 10^{-3}	Rel % Found	Rel % Calcd
Iodinin (**1**)	1562	381	100	100	100	1255	306	100	100
1,6-Phenazinediol (**2**)	1765	374	98. 1	100	100	1430	303	99, 2	100
Pyrazinetetracarboxylic acid (**3**)	1085	278	72, 9	0[c] 50 100	75[d]	782	200	65, 1	66, 6
Pyrazine (**4**)	1135	91	23, 9	0[c] 50 100	25[d]	1310	105	34, 2	33, 3
$BaCO_3$ (**5**)	950	187	49, 1	0[c] 50	50[d]	508	100	32, 8	33, 3

[a] Disintegrations per minute, a unit proportional to counts per minute which includes the counting efficiency. [b] Relative molar activities = dpm × molecular weight (Birch, 1958). These numbers are linearly proportional to the molar activities. [c] All possibilities considered. [d] Joined as in Figure 2.

there for 1 hr, then gradually raised to 215°. Pyrazine distilled from the reaction mixture as a colorless liquid which crystallized immediately. After recrystallization from water (1 ml), pyrazine was obtained as colorless prisms (5 mg), mp 54–55° (lit. mp 55°, Wolff, 1893).

Decarboxylation of Pyrazinetetracarboxylic Acid to Carbon Dioxide (**5**). Pyrazinetetracarboxylic acid (20 mg) and 20 mg of copper chromite were mixed in a microporcelain boat which was then inserted in a horizontal tube. The tube temperature was brought to 290°. The CO_2 resulting from the decarboxylation was removed from the reaction area by a slow stream of oxygen-free N_2 and absorbed by passing the gas serially through three vessels containing 1 N NaOH. The absorbed CO_2 was converted into $BaCO_3$ and recovered by filtration.

Melting points were determined on a Kofler micro hot stage. Absorption spectra were taken on a Unicam-SP 700 machine. Radioactivity of all samples was determined on a low-background apparatus, voltage 1700 V. Samples were counted by using the infinitely thick-layer method on polythene planchets, diameter 6.28 mm; each sample was assayed twice for 5 min. Values of relative molar activities were used for calculations (Birch *et al.*, 1958).

TABLE II: Degree of Incorporation of Labeled Precursors into Iodinin and CO_2.

Compound	Incorp (Rel %) Iodinin	CO_2
[G-^{14}C]Shikimic acid	3.2	7.2
[1-^{14}C]Acetate	0.001	69.1
[2-^{14}C]Acetate	0.05	65.7
[G-^{14}C]Glutamic acid	0.1	68.3

[a] Incorporation (rel %) = 100 (mCi/mmole in isolated product)/(mCi/mmole in ^{14}C compound added).

Results

A summary of the chemical degradation of iodinin is shown in Figure 1. Table I summarizes the results of radioactivity measurements. The distribution of radioactivity among the degradation products after adding DL-[1,6-^{14}C]-shikimic acid clearly indicates the involvement of two molecules of shikimic acid, not one, as C-6 units in iodinin bio-

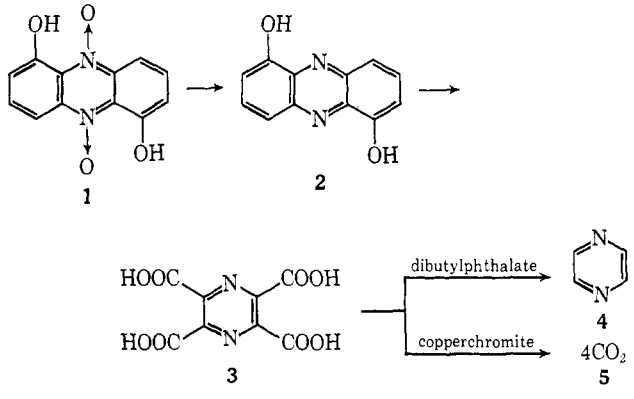

FIGURE 1: Chemical degradation of iodinin. The numbers under each compound correspond to those used in Table I.

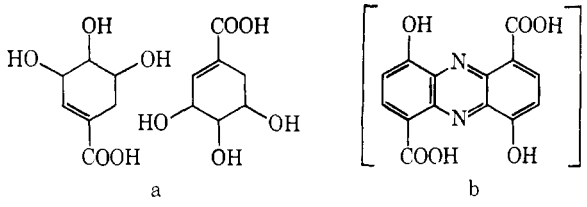

FIGURE 2: (a) Arrangement of 2 shikimic acid units in iodinin. (b) Hypothetical key intermediate common to all natural phenazines.

synthesis. The results with [G-^{14}C]shikimic acid are in accord with either one or two C-6 units.

A comparison of the incorporation of the activity from various substrates into iodinin and respiratory carbon dioxide appears in Table II. The degree of incorporation of shikimic acid into iodinin and the amount of radioactive carbon dioxide obtained show that shikimic acid is not metabolized very efficiently. This indicates that shikimic acid is not degraded extensively prior to incorporation. Acetic and glutamic acids do not contribute significantly to iodinin carbon but are drastically degraded by the organism as shown by the recovery of considerable radioactivity as respiratory carbon dioxide. These findings show that the scrambling of label from shikimic acid was negligible.

Discussion

Many groups of natural products appear to be formed in a related manner, namely, by the synthesis of a specific intermediate product which defines the whole group. This key molecule then undergoes a variety of relatively nonspecific processes (Bu'Lock, 1965; Whalley, 1967). When one considers the structures of the 28 known natural phenazines (see references in Gerber, 1969; Hill and Johnson, 1969) it is apparent that the two shikimic acid units must be arranged as in Figure 2a. This is in accord with the distribution of radioactivity in the degradation products and places the carboxyl groups at positions 1 and 6 of the phenazine ring, an arrangement most clearly illustrated in the griseolutein group of phenazines (Nakamura *et al.*, 1964; Yagishita, 1960). The recent isolation of phenazine-1,6-dicarboxylic acid and ten other phenazines from a bacterium lends additional support (Gerber, 1969). By contrast, for phenoxazinones including actinomycins, the monocyclic units (various substituted 3-hydroxyanthranilic acids) dimerize in a manner that places the two carboxyl groups or their equivalents at positions 1 and 9 of the phenoxazinone ring (Katz, 1967).

By a plausible sequence of reactions from shikimic acid, one can arrive at the hypothetical key phenazine shown in Figure 2b. From it, the structures of all of the known natural phenazines, except aeruginosin B which contains an aromatic sulfonic acid group (Herbert and Holliman, 1969), can be derived using one or more well-known biosynthetic transformations. These include O methylation, N methylation, decarboxylation of phenolic acids, N oxidation, reduction of phenolic hydroxyl, and hydroxylation or amination at a ring site activated for substitution.

Acknowledgments

We thank the Girdler Catalysts Department of Chemetron Chemicals, Louisville, Ky., for copper chromite.

References

Akabori, H., and Nakamura, M. (1959), *J. Antibiot. (Tokyo) Ser. A 12*, 17.

Birch, A. J., Massy-Westropp, R. A., Rickards, R. W., and Smith, H. (1958), *J. Chem. Soc.*, 360.

British Patent 560965 (1946), *Chem. Abstr. 40*, 5074.

Bu'Lock, J. D. (1965), The Biosynthesis of Natural Products, New York, N. Y., McGraw-Hill, pp 8, 32–34.

Carter, R. E., and Richards, J. H. (1961), *J. Amer. Chem. Soc. 83*, 495.

Chang, P. C., and Blackwood, A. C. (1968), *Can. J. Biochem. 46*, 925.

Gerber, N. N. (1969), *J. Heterocycl. Chem. 6*, 297.

Gerber, N. N., and Lechevalier, M. P. (1964), *Biochemistry 3*, 598.

Gerber, N. N., and Lechevalier, M. P. (1965), *Biochemistry 4*, 176.

Herbert, R. B., and Holliman, F. G. (1969), *J. Chem. Soc., C*, 2517.

Hill, J. C., and Johnson, G. T. (1969), *Mycologia 61*, 452.

Hollstein, U., Burton, R. A., and White, J. A. (1966), *Experientia 22*, 210.

Ingledew, W. M., and Campbell, J. J. R. (1969), *Can. J. Microbiol. 15*, 535, 595.

Katz, E. (1967), *in* Antibiotics, Vol. II, Gottlieb, D., and Shaw, P. D., Ed., New York, N. Y., Springer-Verlag, p 316.

Levitch, M. (1961), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 20*, 350.

Levitch, M. E., and Reitz, P. (1966), *Biochemistry 5*, 689.

Levitch, M. E., and Stadtman, E. R. (1964), *Arch. Biochem. Biophys. 106*, 194.

Light, R. J., and Hauser, C. R. (1961), *J. Org. Chem. 26*, 1296.

MacDonald, J. C. (1967), *in* Antibiotics, Vol. II, Gottlieb, D., and Shaw, P. D., Ed., New York, N. Y., Springer-Verlag, p 52.

Nakamura, S., Maeda, K., and Umezawa, H. (1964), *J. Antibiot. (Tokyo), Ser. A, 17*, 33.

Podojil, M., and Gerber, N. N. (1967), *Biochemistry 6*, 2701.

Takeda, R., and Nakanishi, I. (1959), *Hakko Kogaku Zasshi 38*, 9.

Whalley, W. B. (1967), *in* Biogenesis of Natural Compounds, Bernfeld, P., Ed., London, Pergamon, p 1055.

Wolff, L. (1893), *Chem. Ber. 26*, 721.

Yagishita, K. (1960), *J. Antibiot. (Tokyo) Ser. A 13*, 83.