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The Biosynthesis of 1,6-Phenazinediol 5,10-Dioxide (Iodinin) by *Brevibacterium iodinum**

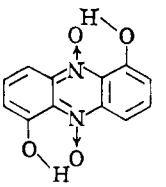
Miloslav Podojil† and Nancy N. Gerber

ABSTRACT: In experiments with resting cells of *Brevibacterium iodinum* the highest yield of iodinin occurred in the presence of some three-, four-, or five-carbon amino acids. Tricarboxylic acid cycle compounds, especially succinic acid, also gave high yields. However, the direct incorporation of labeled glutamic acid into iodinin was only 0.1%, and using growing cultures, the activity was diluted by the addition of compounds of the shikimic acid pathway. The average incorporation of labeled shikimic acid into iodinin was 3.7%. This activity was diluted by L-phenylalanine but not significantly by other amino acids or by fumaric acid. Anthranilic acid is not a direct precursor of iodinin. Labeled 1,6-phenazinediol and 1,6-phenazinediol 5-oxide were incorporated into iodinin in high efficiency (10 and 15%), confirming the idea that these two substances are the immediate biosynthetic precursors of iodinin.

The relatively good incorporation of shikimic acid into iodinin indicates that the pathway for the biosynthesis of iodinin is similar to that of the phenazines studied by others.

About 20 naturally occurring phenazines are known at present, all produced by microorganisms. The biosynthesis of four of them has been studied. Using labeled substrates to study the formation of pyocyanine (1-hydroxy-5-methylphenazinium betaine) by *Pseudomonas aeruginosa*, Blackwood and Neish (1957) showed the incorporation of glycerol and dihydroxyacetone; Frank and DeMoss (1959) of L-alanine, pyruvate, and glycerol; Ingram and Blackwood (1962) of glycerol, alanine, and leucine; Millican (1962) of shikimic acid; and MacDonald (1963a), of glycerol, shikimic, and quinic acids. Sheikh and MacDonald (1964) demonstrated that the 5-methyl group was derived from methionine. When unlabeled substances were used for the production of pyocyanine some amino acids and some tricarboxylic acid cycle compounds with ammonium ions as a source of nitrogen were found to be the most efficient (Grossowicz *et al.*, 1957; Valette *et al.*, 1964). Carter and Richards (1961) reported the incorporation of [carboxyl-^{14}C]anthranilic acid into chlororaphin (a 3:1 molecular compound of phenazine-1-carboxamide and its 5,10-dihydro derivative) produced by *Pseudomonas chlororaphis*. Levitch (1961) showed that DL-[3-^{14}C]tryptophan and/or [1,3-^{14}C]glycerol were precursors of phenazine-1-carboxylic acid in *P. aureofaciens*. Later, labeled shikimic acid was shown to be incorporated into phenazine-1-carboxylic acid (Levitch and Stadtman, 1964) and 2-hydroxyphenazine (Levitch and Rietz, 1966). Acetate was demonstrated to be a much less efficient phenazine precursor (Blackwood and Neish, 1957; Levitch, 1961; MacDonald, 1963b; Levitch and Stadtman, 1964).

Since no member of the important group of 1,6-dioxygenated phenazines had been studied, we decided to investigate the biosynthesis of iodinin. Produced by *Brevibacterium iodinum* (Clemo and McIlwain, 1938; Clemo and Daglish, 1950) this extremely insoluble pigment which, in bulk, looks like iodine was the first naturally occurring *N*-oxide known. It has antimicrobial (Gerber and Lechevalier, 1964; Oda *et al.*, 1966) and antitumor activity (Makino *et al.*, 1963; L. H. Pugh, unpublished data). In *B. iodinum*, *Microbispora*



* From the Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey. *Received September 27, 1966.* The U. S. Public Health Service Grant AI 06230-02 supported this investigation. The microorganism used in these studies has been previously referred to as *Pseudomonas iodina* and *Chromobacterium iodinum*. Sneath (1956) observed that it is a Gram-positive diptheroid bacterium. It is probably best included with the brevibacteria.

† Waksman–Merck Postdoctoral Fellow. On leave of absence from the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague.

aerata, and *Streptomyces thioluteus* iodinin is accompanied by 1,6-phenazinediol and 1,6-phenazinediol 5-oxide (Gerber and Lechevalier, 1965).

Materials and Methods

B. iodinum no. 26 received from Dr. R. E. Gordon of this Institute was used in these investigations. It was maintained on YD slants (10 g of Difco yeast extract, 10 g of Cerelose, 15 g of agar/l., pH 7.0–7.2), transferred every 4 weeks, incubated at 28°, and when well grown stored at 5°. Cultures used for experiments were always less than 2-weeks old.

DL-[3-^{3}H]Glutamic acid (sp act. 5 c/mmole) and [G-^{14}C]shikimic acid (sp act. 3.36 mc/mmole) were purchased from New England Nuclear Corp. Tritium-labeled 1,6-phenazinediol (sp act. 10.5×10^2 dpm/mmole) and 1,6-phenazinediol 5-oxide (sp act. 3.85×10^2 dpm/mmole) were prepared biosynthetically using DL-[3-^{3}H]glutamic acid and 36-hr-old shaken cultures of *B. iodinum* (Gerber and Lechevalier, 1965).

Phenazine-1-carboxylic acid was prepared according to Kögl and Postowsky (1930) holding the temperature at 165° for 10 min. One-fourth of the concentrated chloroform extract was chromatographed on a 100-g column of Mallinckrodt silicic acid (SilicAR CC-7, 100–200 mesh). After development with chloroform, the middle fractions contained the desired product, mp 244–245°. Phenazine-1,6-dicarboxylic acid was synthesized by the method of Birkofer and Widmann (1953). It gave a red solution in concentrated sulfuric acid and blackened at 315–320° without melting.

The conditions of cultivation and isolation of metabolites have been described (Gerber and Lechevalier, 1965). All figures given in the tables are the average of duplicate runs. In experiments with resting cells, 24-hr-old cultures were centrifuged 10 min at 6000 rpm. The solids were washed with three portions of cold saline–0.05 M phosphate buffer (pH 7) (1:1) equal to the original volume, and the white cells were separated mechanically from the dark iodinin crystals. About 500 mg (wet weight) of cells was added to 50 ml of saline–phosphate buffer (pH 8) (1:1) or "salts E" solution (Frank and DeMoss, 1959). After the addition of substrate, the pH was adjusted to 8.0 if necessary and incubation was continued 24 hr with shaking.

In experiments with growing cultures labeled compounds were added to 36-hr-old cultures unless otherwise noted. Incubation was continued for 24 hr with tritium-labeled substrates and for 48 hr with [^{14}C]-shikimic acid. Radioactive iodinin was purified by column chromatography on silicic acid then assayed spectrophotometrically in chloroform.

The radioactivity of all samples was determined in a Model 574 Tri-Carb liquid scintillation spectrometer (Packard Instruments Co., LaGrange, Ill.), using 15 ml of a toluene solution containing 5 g/l. of 2,5-diphenyloxazole and 0.3 g/l. of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene in a 20-ml glass vial. The figures listed in the table are the average of two or three 10-min counts, and are expressed as disintegrations per minute in order to include the counting efficiency (26% for tritium and 30% for ^{14}C) which was determined by automatic external standardization.

Results

Experiments with Unlabeled Compounds. In the experiments with washed cells, the utilization of various compounds for iodinin production was studied. The results are shown in Table I. In addition no iodinin was formed when the following substances were added (all at 500 μmoles/flask except as noted): DL-α-aminopimelic acid, DL-α,ε-diaminopimelic acid, DL-α-aminoadipic acid, D-glutamine, D-asparagine, DL-threonine, sarcosine, quinic acid (and 500 μmoles of

TABLE I: The Effect of Added Substances on Iodinin Production by Washed Cells.

Substrate (500 μmoles/flask)	Iodinin Produced[a] in Saline–Phosphate Buffer	Iodinin Produced[a] in Salts E Soln
None (control)	0	0
ε-Aminocaproic acid	7	6
DL-α-Aminocaproic acid	10	7
L-Lysine	5	5
L-Leucine	15	17
L-Isoleucine	12	20
L-Arginine-HCl	44	35
L-Glutamic acid	51	25
DL-Glutamic acid	40	21
D-Glutamic acid	9	1
L-Glutamine	100	56
L-Ornithine	27	32
L-Aspartic acid	15	9
D-Aspartic acid	0	0.5
L-Asparagine	70	72
DL-Asparagine	0.2	0.9
L-Alanine	25	68
L-Serine	3	46
Glycine	0.6	0
Oxalacetic acid[b]	30	34
Citric acid	1.2	0.5
α-Ketoglutaric acid	14	3.4
Succinic acid	56	90
Fumaric acid	67	15
Malic acid	34	50
Glycerol	16	20
Shikimic acid	9	8
L-Phenylalanine	25	38

[a] Expressed relative to the amount of iodinin produced (6.5 μmoles/flask) with L-glutamine in saline–phosphate buffer. [b] With all nonnitrogenous substrates, 500 μmoles of ammonium chloride was added.

TABLE II: Incorporation of Labeled Substances into Iodinin.

Compound	Total Act. (dpm)	Iodinin Isolated (μmole)	Iodinin Total Act. (dpm × 10^3)	% Incorp
DL-[3-^{3}H]Glutamic acid[a]	2.2×10^7	0.65	25	0.11
DL-[3-^{3}H]Glutamic acid[b]	2.2×10^7	4.6	2.3	0.01
DL-[3-^{3}H]Glutamic acid[c]	2.2×10^7	11	7.1	0.03
[^{3}H]1,6-Phenazinediol	21×10^2	4.1	0.21	10
[^{3}H]1,6-Phenazinediol 5-oxide	15×10^2	4.8	0.22	15
[G-^{14}C]Shikimic acid[d]	2.2×10^6	4.3	63	2.9
[G-^{14}C]Shikimic acid[e]	1.1×10^6	7.3	43	3.9
[G-^{14}C]Shikimic acid[e]	1.1×10^6	8.8	49	4.4

[a] Labeled glutamic acid (10 μc) and carrier (100 μmoles) added to washed cells in saline–phosphate buffer. [b] Labeled substrate (10 μc) and carrier (10 μmoles) added to growing cultures at 36 hr. [c] Same as *b* but at 72 hr. [d] Labeled shikimic acid (1 μc) and carrier (10 μmoles) added to growing cultures at 36 hr. [e] Same as *d* but 0.5 μc.

TABLE III: Effect of Added Substances on the Incorporation of DL-[3-^{3}H]Glutamic or [G-^{14}C]Shikimic Acids into Iodinin.

Substance Added	Amt Added (μmole/50 ml)	Iodinin Produced (μmole)	Total Act. of Iodinin (dpm × 10^3)	% Decrease (−) or Increase (+) of Act.
None[a]	5	6	2.1	0
Phenazine	5	4.3	1.0	−52
1,6-Phenazinediol	5	6.0	0.63	−70
Phenazine-1,6-dicarboxylic acid	5	5.2	1.0	−52
Shikimic acid	50	9.0	1.7	−18
Quinic acid	50	7.5	1.5	−26
DL-Tryptophan	50	5.8	1.0	−52
L-Phenylalanine	50	5.5	1.4	−23
DL-Glutamic acid	50	5.7	1.0	−48
L-Glutamic acid	50	5.0	0.96	−63
DL-α,ε-Diaminopimelic acid	50	5.9	2.0	−4
Fumaric acid	50	5.5	1.2	−40
None[b]		7.3	43	0
DL-Glutamic acid	50	7.6	41	−5
L-Leucine	50	6.8	42.5	−1
L-Arginine	50	6.9	37	−14
L-Alanine	50	7.1	38.5	−10
Fumaric acid	50	6.8	36.5	−15
L-Phenylalanine	50	8.5	18	−58
Anthranilic acid	25	5.9	72	+60

[a] In the experiments reported in the upper half of the table the flasks contained 10 μc of labeled glutamic acid and 10 μmoles of carrier. [b] In the lower half of the table, 0.5 μc of labeled shikimic acid and 10 μmoles of carrier.

NH_4Cl as a nitrogen source), L-tryptophan, DL-tryptophan, and anthranilic acid (100 μmoles/flask). In saline–phosphate buffer, L-glutamine, L-asparagine, and succinic and fumaric acids furnished the highest yield of iodinin; in salts E solution, L-asparagine, L-glutamine, L-alanine, and succinic and malic acids were the most effective. Generally, L-amino acids were more efficient than the D or DL forms; however, the absolute yields of iodinin in all cases were low. Tricarboxylic acid cycle compounds were utilized but shikimic acid

pathway compounds poorly or not at all. Some hypothetical iodinin precursors containing the phenazine ring were not utilized for iodinin production. These were phenazine, phenazine-1-carboxylic acid, and phenazine-1,6-dicarboxylic acid (5 μmoles/flask).

Experiments with Labeled Compounds. A. DIRECT INCORPORATION. The results are shown in Table II. Glutamic acid was incorporated into iodinin with low efficiency by resting cells and whole cultures. Labeled 1,6-phenazinediol and its 5-oxide were both incorporated extensively. Shikimic acid was incorporated about 300 times more than glutamic acid under the same conditions.

B. RADIOACTIVE DILUTION EXPERIMENTS. The direct incorporation experiments were confirmed and extended by isotope dilution studies summarized in Table III. Although shikimic acid, quinic acid, and DL-tryptophan, as well as phenazine and phenazine-1,6-dicarboxylic acid, were not used directly for iodinin production by washed cells (Table I), they did dilute the activity of iodinin biosynthesized from labeled glutamic acid. Added aliphatic amino acids or fumaric acid affected the activity of iodinin from shikimic acid only slightly. However, phenylalanine lowered the activity by 58% and anthranilic acid increased it by 60%.

Discussion

The experiments with resting cells (Table I) indicated that a variety of amino acids could serve as precursors of iodinin. This idea was supported by the dilution of labeled glutamic acid when amino acids were added (Table III). The relatively higher yield of iodinin from amino acids of three to five carbon atoms could be explained by better penetration of these molecules through the bacterial membrane. One might suppose that two molecules of amino acid could condense to form a dioxopiperazine. Modifications of such structures are known among microbial products (aspergillic, hydroxyaspergillic, and pulcherrimic acids (MacDonald, 1961, 1962, 1963a,b)) and 3,6-dibenzylidine-2,5-dioxopiperazine has been isolated from two iodinin-producing organisms, *S. thioluteus* (N. N. Gerber, unpublished data) and a novel strain of *Nocardiaceae* (Gerber, 1966). The two aliphatic side chains of the dioxopiperazine after appropriate extension and modification might cyclize to form the two end rings of a phenazine.

However, the much higher direct incorporation of labeled shikimic acid than of glutamic acid into iodinin and the failure of amino acids to dilute this incorporation appreciably ruled out such a direct participation for these aliphatic amino acids. Rather, amino acids must be involved after extensive modification to other intermediates. The role of tricarboxylic acid cycle compounds appears to be similar. The efficient ones are those easily transformed to amino acids. The relatively good incorporation of shikimic acid into iodinin indicated that the pathway for the biosynthesis of iodinin is similar to that of pyocyanine Millican, 1962; MacDonald, 1963a,b), phenazine-1-carboxylic acid (Levitch and Stadtman, 1964), and 2-phenazinol (Levitch and Rietz, 1966). The dilution of activity by other compounds tested was negligible except for L-phenylalanine. On the other hand, anthranilic acid enhanced the total activity of iodinin and lowered its yield. Both of these compounds are involved in the shikimic acid pathway but in different branches (Gibson *et al.*, 1962). Thus it seems unlikely that anthranilic acid is a true precursor of iodinin. Anthranilic acid may block reactions connected with either the synthesis or transformations of shikimic acid or some related intermediate. In addition, the slightly lower yield of iodinin may indicate inhibition of an enzyme necessary for the conversion of the intermediate to a phenazine.

The extensive incorporation of active 1,6-phenazinediol and its 5-oxide shows that these are direct precursors of iodinin as has already been suggested (Gerber and Lechevalier, 1965). In contrast, Frank and DeMoss (1959) failed to find any radioactive pyocyanine after adding labeled 1-phenazinol. The role of phenazine and phenazine-1,6-dicarboxylic acid (Tables I and III) as possible precursors of iodinin is not clear. In experiments with resting cells, the failure to produce iodinin may have been due to difficiencies in enzyme contact under the conditions used.

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Mechanisms of Synthesis of Waxy Esters in Broccoli (*Brassica oleracea*)*

P. E. Kolattukudy

ABSTRACT: Broccoli (*Brassica oleracea*) leaf is shown to contain enzymes capable of synthesizing waxy esters from fatty alcohols by direct esterification with fatty acids and by an acyl transfer from phospholipids and acyl coenzyme A (acyl-CoA). Young broccoli leaves readily incorporated [U-^{14}C]stearyl alcohol mostly into waxy esters. The mechanism of esterification was studied with acetone powder prepared from the leaves. This material readily incorporated labeled stearyl alcohol (C_{18}) and cetyl alcohol (C_{16}) (but not cholesterol) into esters by utilizing endogenous acyl compounds. Radio gas–liquid partition chromatography of the esters showed that the major endogenous acyl moiety was a palmityl group (C_{16}). The pH optimum was 5.0, and half the maximal rate was obtained with 2.5×10^{-5} M stearyl alcohol with the acetone powder suspension. Bovine serum albumin inhibited the reaction, and this inhibition could be partially reversed by free fatty acids which when added alone showed some inhibition. Neither adenosine triphosphate (ATP) and CoA supplied with palmitic acid nor palmityl-CoA stimulated esterification of [^{14}C]stearyl alcohol. Significant incorporation of [U-^{14}C]palmitic acid into the ester suggested the occurrence of direct esterification of free fatty acids with stearyl alcohol. The [^{14}C]-palmityl moiety of exogenous phospholipids (and triglycerides) was much more efficiently incorporated into waxy ester than would be expected from the quantity of free ^{14}C fatty acids produced from them, suggesting the occurrence of an acyl transfer from a phospholipid (and triglyceride) to the fatty alcohol. The relative participation of direct esterification in wax synthesis increased when the concentration of fatty alcohol was increased. About one-third of the esterifying activity of the acetone powder was soluble in buffer, and most of this activity was precipitated at 25–60% saturation with $(NH_4)_2SO_4$. Palmityl-CoA served as a substrate for esterification with this fraction. The ammonium sulfate precipitated enzyme was further purified by Sephadex G-100 gel filtration by which fatty acid binding proteins could be separated from the enzyme. The gel filtration increased the stimulation of esterification caused by palmityl-CoA, and the enhanced esterification produced mainly stearyl palmitate. The esterification reaction that required acyl-CoA had a broad pH optimum above 6.0, and the reaction was inhibited by bovine serum albumin and to a lesser extent by free fatty acids. The major part of the esterifying activity of broccoli leaf homogenate was located in the soluble proteins, but this system was not stimulated by addition of ATP, CoA, and palmitic acid, or of palmityl-CoA.

Waxy esters constitute one of the most common class of compounds found in the surface lipids of plants (Douglas and Eglinton, 1965; Martin, 1964; Silva Fernandes *et al.*, 1964), animals (Nicolaides, 1965), and insects (Baker *et al.*, 1960; Gilby and Cox, 1963). Certain unusual oils such as oil from the head of sperm whale (spermaceti), castor oil fish (*Ruvettus pretiosus*) (Cox and Reid, 1932), "Mutton bird oil" (Carter, 1921), and Mullet (*Mugil cephalus*) roe oil (Iyengar and Schlenk, 1967) contain waxy esters as the major component. The alcohol and acid moieties usually range in chain length from C_{10} to C_{30}, and carbon chains with an even number of carbon atoms predomi-

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