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# The Biosynthesis of Hadacidin*

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ABSTRACT: Glycine, formate, and the β-carbon of serine were all found to be very rapidly incorporated into hadacidin during its synthesis by *Penicillium aurantio-violaceum*. Degradation of hadacidin showed that C-1 of glycine was found almost exclusively in the glycyl portion of hadacidin while formate, the β-carbon of serine, and the α-carbon of glycine were incorporated into both the glycyl and formyl portions of the hydroxamate. *N*-Hydroxyglycine was incorporated into hadacidin at a rate equal to that for glycine in 3-hr periods and to a much greater extent in longer time periods. *N*-Hydroxyglycine, but not glycine, brought about a net stimulation of hydroxamate production. Nitroacetic acid, glyoxylic acid oxime, and formylglycine were not rapidly incorporated into hadacidin. Experiments with $^{18}O$ showed that the hydroxylamino oxygen atom of hadacidin is derived from oxygen gas rather than water. The experimental results are consistent with the hypothesis that the biosynthesis of hadacidin occurs by *N*-oxygenation of glycine to yield *N*-hydroxyglycine followed by *N*-formylation to yield the hydroxamate.

Nitrogen at the oxidation state of −1 is found in biological systems as the hydroxylamino group of hydroxamic acids of fungal origin. The hydroxylamino group is frequently donated by a hydroxylamino acid (*N*-hydroxyamino acid), such as δ-*N*-hydroxyornithine found in the siderochromes (Keller-Schierlein *et al.*, 1964). Tracer studies using labeled amino acids have been carried out with aspergillic acid (MacDonald, 1961), mycelianamide (Birch and Smith, 1958), and ferrichrome (T. F. Emery, unpublished). In every case it was found that the carbon skeleton of the amino acid is incorporated into the hydroxylamino acid portion of the hydroxamic acid. Nothing is known, however, about the mechanism of formation of the hydroxamate group itself.

Hadacidin, or *N*-formyl-*N*-hydroxyglycine, is the simplest known naturally occurring hydroxamic acid. This compound, isolated and characterized by Kaczka *et al.* (1962), seemed well suited for a study of the route of hydroxamic acid biosynthesis. The hydroxamate bond may be considered to be a peptide bond with an oxygen atom on the amide nitrogen, but there is no *a priori* reason to decide whether the oxygen atom is introduced before or after the formation of the amide bond. In the latter case, formylglycine would be an intermediate in hadacidin biosynthesis. *N*-Hydroxylation of an amide bond was reported by Cramer *et al.* (1960), who found that *N*-hydroxy-2-acetylaminofluorene was formed in the intact rat upon administration of 2-acetylaminofluorene. Nevertheless, this finding cannot be considered direct proof of *N*-hydroxylation of an amide bond because, as the authors point out, the acetyl group is labile in their experiments, and hydroxylation of the amino group might have occurred.

Formation of the hydroxylamino group prior to amide bond formation, that is, at the level of the free amino acid, might occur by several routes. (A) DIRECT OXIDATION OF AN AMINO GROUP. No such reaction has been described in biological systems, although Baker and Chaykin (1960) observed a reduced triphosphopyridine nucleotide dependent oxidation of trimethylamine to trimethylamine *N*-oxide, and the *N*-oxide of *N,N*-dimethyltryptamine can be formed by mouse liver homogenates (Fish *et al.*, 1955). Oxidation of aniline to nitrosobenzene has also been reported (Böttcher and Kiese, 1960). However, hydroxylamino acids have not been found free even in hydroxamate synthesizing organisms.

(B) REDUCTION OF A NITRO GROUP. Nitro groups in compounds of biological origin are very rare. Enzymic reduction of an aromatic nitro group yields the amine (Zucker and Nason, 1955), although in one instance 4-hydroxylamino-2,6-dinitrotoluene was found as the product of enzymic reduction of trinitrotoluene (Bueding and Jolliffe, 1946). 1-Amino-5-nitropentane stimu-

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lates formation of the ferrioxamine-type hydroxamic acids, which contain 1-amino-5-hydroxylaminopentane (V. Prelog, personal communication), and we were thus led to consider nitroacetic acid as a possible hadacidin precursor.

(C) 2-ELECTRON REDUCTION OF AN OXIME. The oximes of several keto acids have been isolated from *Torulopsis utilis* (Virtanen and Saris, 1956), and enzymic formation of oximes has been reported (Yamafuji and Omura, 1957). The biological significance of these oximes is not known nor has any reduction of oximes to hydroxylamino compounds been described. Nevertheless, oxime formation *via* the condensation of hydroxylamine with a keto acid would provide a general route of hydroxylamino acid biosynthesis, and we therefore considered glyoxylic acid oxime in our experiments.

(D) HYDRATION OF AN IMINO ACID.

(E) ADDITION OF HYDROXYLAMINE TO A DOUBLE BOND. The condensation of hydroxylamine with fumaric acid to yield *N*-hydroxyaspartic acid is the only known enzymic formation of a hydroxylamino acid (Emery, 1963). Such a reaction clearly cannot be invoked as a general route of hydroxylamino acid synthesis since *N*-hydroxyglycine formation is not possible by such a mechanism.

With these considerations in mind, experiments were performed to elucidate the route of hadacidin biosynthesis in *Penicillium aurantio-violaceum*. The results described in this paper suggest that direct oxidation of glycine to yield *N*-hydroxyglycine (A), followed by formylation of the hydroxylamino group, is the pathway of hydroxamate biosynthesis in this organism.

## Experimental Procedures

*Materials.* [1-$^{14}$C]Glycine, [2-$^{14}$C]glycine, [$^{14}$C]sodium formate, and DL-[3-$^{14}$C]serine were obtained from the New England Nuclear Corp. $H_2{}^{18}O$ was obtained from the Weizmann Institute of Research, Rehovoth, Israel.

Formylglycine was synthesized by a procedure adapted from Biilman *et al.* (1934). Equimolar quantities of formic acid and acetic anhydride were mixed and added in 20% excess to glycine dissolved in a minimal amount of formic acid. After 1 hr the solution was concentrated to dryness, the residue was dissolved in water, and the solution again was concentrated to dryness. The residue was dissolved in hot water and crystals formed upon cooling, mp 148–150° (lit. 152–153°).

*Anal.* Calcd for $C_3H_5NO_3$: C, 34.96; H, 4.89; N, 13.59. Found: C, 34.96; H, 4.99; N, 13.80.

The compound gave one spot when subjected to paper chromatography and paper electrophoresis. Formylglycine was detected on the paper by the chlorine–starch–iodide test (Rydon and Smith, 1952). Carboxyl-labeled formylglycine was synthesized by the same procedure using [1-$^{14}$C]glycine. Doubly labeled formylglycine was synthesized from [$^{14}$C]sodium formate and [1-$^{14}$C]glycine.

*N*-Hydroxyglycine was produced by a slow hydrolysis of hadacidin (Kaczka *et al.*, 1962). Radioactive *N*-hydroxyglycine (30,000 cpm/mg) was made from hadacidin produced biosynthetically by growing the organism in the presence of [1-$^{14}$C]glycine. The *N*-hydroxyglycine was free of glycine, hadacidin, and formylglycine as determined by electrophoresis at pH 6.0 using pyridine–acetic acid–water, 109:10:1884. The *N*-hydroxyglycine was detected as a pink spot by the tetrazolium spray of Snow (1954) or by a brown color with ninhydrin; mp 146–148° (lit. 145–150°).

*Anal.* Calcd for $C_2H_5NO_3$: C, 26.37; H, 5.53; N, 15.38. Found: C, 26.26; H, 5.67; N, 15.29.

Disodium nitroacetate was synthesized according to the procedure of Steinkopf (1909). Nitroacetate was reduced in 16 hr by hydrogen at 50 psi in the presence of Adams' catalyst yielding glycine as the sole product. Disodium nitroacetate was difficult to dry, and no satisfactory elemental analysis was obtained. Glyoxylic acid oxime was synthesized from glyoxylic acid and hydroxylamine by the procedure of Wieland (1910); mp 137–138° (lit. 137–138°).

*Anal.* Calcd for $C_2H_3NO_3$: C, 26.98; H, 3.39; N, 15.73. Found: C, 26.72; H, 3.64; N, 15.12.

The compound sublimes when heated at 100° *in vacuo*. Glyoxylic acid oxime was reduced under the same conditions as used for the nitroacetic acid. Paper electrophoresis and paper chromatography showed the reduction to be complete, glycine being the sole product. Glyoxylic acid oxime gives no ninhydrin color but yields a white spot on a dark background when sprayed with a 1% solution of titanium trichloride.

*Methods. Penicillium aurantio-violaceum* (MF-4070) was grown on slants composed of dextrose, 1%; Edamine (enzymic digest of lactalbumin), 0.5%; yeast extract, 1%; and agar, 2% (Gitterman *et al.*, 1962). After 7–10 days of growth at 26°, 5 ml of sterile salt solution (Davis and Mingioli, 1950) was added to each slant. The spores were scraped free and 1.5 ml of spore suspension was used to inoculate 100 ml of production medium. The production medium contained glucose, 4%; corn steep liquor, 1%; and Edamine, 2%. The pH was adjusted to 6.8 with 1 N NaOH prior to sterilization. Each 500-ml erlenmeyer flask contained 100 ml of medium and was incubated at 26° on a rotary shaker moving at 220 rpm. All isotope incorporation experiments were carried out under these conditions unless otherwise stated.

*Isolation and Characterization of the Monosodium Salt of Hadacidin.* The procedure of isolation and purification of hadacidin was that used by Kaczka *et al.* (1962). In this paper the monosodium salt of hadacidin will be referred to as hadacidin. Occasionally the isolation procedure was modified by adding activated charcoal (Nuchar C-190N) to the methanol extract to remove orange color. All samples of hadacidin were dried for at least 2 hr at 100° over $P_2O_5$ *in vacuo*. The following criteria were used to characterize hadacidin. (1) Melting point: hadacidin (monosodium salt) decomposes at 205–210°.

(2) Paper electrophoresis: hadacidin was spotted on Whatman No. 1 paper, and a potential of 26 v/cm (30 ma) was applied for 60 min. The buffer was pyridine–acetic acid–water, 14:10:930, pH 5.0. Hadacidin,

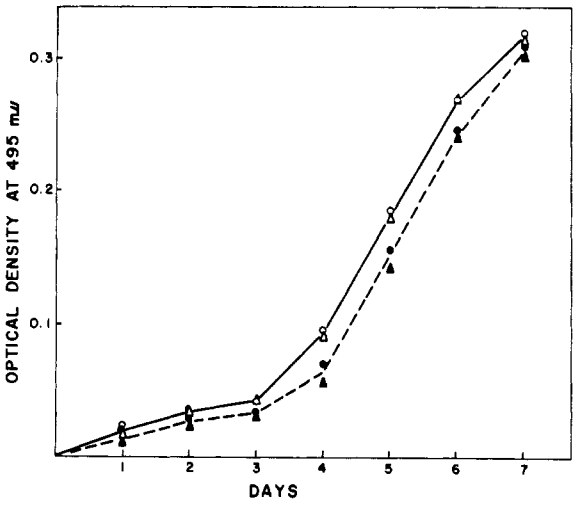


FIGURE 1: Hadacidin production by *P. aurantio-violaceum*. See experimental section for growth conditions and method of assay. The two curves represent flasks inoculated (in duplicate) from two different agar slants of the organism.

which moved toward the anode with an $I_t$ of 10.2 cm, was detected by spraying the dried paper with 3% ferric chloride. The electropherograms were scanned for radioactivity in a Packard Model 7200 radiochromatogram scanner. All of the radioactivity was found in the area of hadacidin.

(3) Spectrophotometry: hadacidin forms a purple complex with ferric ion at pH 2.8 in the presence of a 10-fold excess of ferric chloride. Under these conditions the iron chelate of purified hadacidin shows a maximum absorption at 495 mμ with $E^{1\%}_{1cm}$ 58. Optical measurements were performed using the Beckman DU and Bausch and Lomb Spectronic 505 spectrophotometers.

(4) Reduction: to 25 mg of hadacidin dissolved in a few milliliters of water was added about 100 mg of freshly activated Raney nickel. Hydrogenation was performed at 50 psi for 10 hr. The product of reduction was established as formylglycine by paper electrophoresis and chromatography.

The elemental analysis of a sample of recrystallized hadacidin (monosodium salt) gave: *Anal.* Calcd for $C_3H_4NNaO_3$: C, 25.54; H, 2.86; N, 9.93. Found: C, 25.73; H, 2.97; N, 9.71.

Hadacidin in the culture medium was quantitatively determined by the colored complex it forms with ferric chloride. After centrifugation of the mycelia, 0.1 ml of the supernatant fluid was added to 0.1 ml of 10% $FeCl_3 \cdot 6H_2O$ plus 9.8 ml of distilled water. The solutions were read against a ferric chloride blank at 495 mμ in the Beckman DU using cuvets with 1.0-cm path length. Paper electrophoresis and paper chromatography showed hadacidin to be the only ferric chloride positive substance in the supernatant liquid. Only about 40% of the hadacidin could actually be isolated and crystallized, and all isotope incorporation values were based on the amount of hadacidin present as determined by the ferric chloride assay.

*Degradation of Hadacidin.* Hadacidin and formylglycine were hydrolyzed by dissolving 10–20 mg of the compound in 2 ml of 25% *p*-toluenesulfonic acid and heating in a boiling water bath for 3 hr (Steyermark, 1961). The formic acid distilled at 101° by adding water to the reaction flask and connecting it to a microdistillation apparatus. Degradation controls were run on mixtures of formate plus [1-$^{14}$C]glycine, [$^{14}$C]*N*-hydroxylglycine, and formylglycine plus [$^{14}$C]formate. The $^{14}$C in *N*-hydroxyglycine and glycine was found in the reaction flask and the [$^{14}$C]formate was found in the distillate, recoveries of all $^{14}$C compounds being at least 98%.

*Radioactivity Measurements.* The radioactivity of all $^{14}$C compounds was determined in a Technical Measurement Corp. LP-2A liquid phosphor counter. The compounds were dissolved in 0.5 ml of water and added to 15 ml of scintillation fluid which was made from 4.2 g of 2,5-diphenyloxazole, 52.5 mg of 2,2-*p*-phenylene-bis-5-phenyloxazole, 1 l. of toluene, and 500 ml of absolute ethanol. The efficiency of the counter for $^{14}$C was approximately 40%. Radioactive samples of hadacidin obtained from experiments with less than 5% incorporation of the labeled precursor were counted to a standard deviation of ±7% of the detected counts. Radioactive samples of hadacidin from experiments with greater than 10% incorporation of the labeled precursor were counted to a standard deviation of ±2% of the detected counts.

*Procedures Used in $^{18}O$ Experiments.* Oxygen gas enriched in $^{18}$O was produced by electrolysis of $H_2{}^{18}O$. The oxygen was collected in an inverted bottle by displacement of water layered with paraffin oil to prevent gas exchange between the $^{18}O_2$ generated and dissolved gases in the water. The $^{18}O_2$ experiments were performed by growing the organism in 100 ml of sterile medium in a 500-ml erlenmeyer flask fitted with a center well and side arm. After 3 days of growth, 7 ml of 90% potassium hydroxide was added to the center well, the neck of the flask was stoppered, and the side arm was connected to the supply of $^{18}O_2$. A continual supply of $^{18}O_2$ under a hydrostatic pressure of 18 cm of water was administered from the $^{18}O_2$ reservoir. The center-well potassium hydroxide and the reservoir of $^{18}O_2$ were changed three times during the 3-day experiment. This manipulation took only 15 min to complete, thus exposing the organism to normal air for a negligible time period. Before each reservoir of $^{18}O_2$ was attached, the culture flask and connecting tubes were evacuated to 35 mm and filled with $N_2$. This process was repeated five times prior to introduction of the $^{18}O_2$. In this manner an atmosphere of 95% oxygen enriched with 1.365 atoms per cent excess $^{18}$O was obtained. The oxygen system was patterned after that of Yamamoto *et al.* (1962).

The $^{18}O_2$ sample analyzed was obtained from the $^{18}O_2$ supply line after 24 hr of incubation. In the $H_2{}^{18}O$ experiment an aliquot of the *P. aurantio-violaceum* culture fluid was removed after the third day and distilled at

TABLE I: Incorporation of Glycine, Formate, and Serine into Hadacidin by Cultures of *P. aurantio-violaceum*.

| Compound | Incorporation Time (hr) | Amount Added ($\mu$curies) | Specific Activity (mcuries/mmole) | % Incorporation into Hadacidin |
|---|---|---|---|---|
| [1-$^{14}$C]Glycine | 0.5 | 0.8 | 5.4 | 18 |
| | 1 | 0.8 | 5.4 | 27 |
| | 3 | 0.25 | 0.005 | 47 |
| [$^{14}$C]Sodium formate | 0.5 | 1.3 | 5.0 | 63[a] |
| | 1 | 2.8 | 5.0 | 61[a] |
| | 3 | 2.8 | 5.0 | 59 |
| DL-[3-$^{14}$C]Serine | 0.5 | 2.6 | 5.8 | 60[b] |
| | 1 | 2.4 | 5.8 | 62[b] |
| | 3 | 8.8 | 5.8 | 47[b] |

[a] Average of two experiments. [b] Incorporation values are based on the assumption that only the L-isomer is utilized by the organism.

100°. The distillate ($H_2{}^{18}O$) was kept at −15° until sent for isotopic analysis.

Hadacidin from the $H_2{}^{18}O$ and $^{18}O_2$ experiments was reduced to formylglycine as follows. About 35 mg of hadacidin was dissolved in 4 ml of water, and to this solution was added 0.2 ml of a suspension of freshly activated Raney nickel in ethanol. The solution was hydrogenated at 50 psi for 11 hr. The solution was tested for complete reduction by the ferric chloride test and was then lyophilized. The purity of the formylglycine was examined by paper electrophoresis.

Controls no. 1 and no. 2 were designed to test the possibility of $^{18}O$ exchange between $H_2{}^{18}O$ and hadacidin, and $H_2{}^{18}O$ and formylglycine, respectively. For control no. 1, 42 mg of hadacidin was dissolved in 3 ml of $H_2{}^{18}O$ for 22 hr at 5°. The solution was lyophilized and the hadacidin was dried prior to analysis. The conditions of this control are comparable to those used for crystallization of hadacidin. For control no. 2, hadacidin was reduced to formylglycine under conditions identical with the reduction of $^{18}O$ hadacidin with the exception that $H_2{}^{18}O$ was substituted for water.

Compounds were analyzed for $^{18}O$ content by the Analytica Corp., New York, N. Y. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. All melting points are uncorrected.

## Results

The time course of hadacidin synthesis by *P. aurantio-violaceum* is shown in Figure 1. From the fourth to the seventh day it can be seen that the rate of synthesis of hadacidin is nearly the same in each of the four flasks. During this period the organism produces hadacidinat an average rate of 1 mg/ml per day under growth conditions used in these experiments. All subsequent experiments were performed within this 3-day period of rapid hadacidin synthesis. In all cases where direct comparison of various substrates was made, the culture flasks were inoculated from the same slant. After 8 days of growth the rate of synthesis of hadacidin diminished and remained at a low value for a subsequent 7-day period.

Tracer studies using [1-$^{14}$C]glycine (Table I) indicated that this amino acid is readily incorporated into hadacidin. The data also show that formate and the $\beta$-carbon of serine are incorporated very rapidly and efficiently into hadacidin. In view of the extraordinarily high rate of incorporation of formate and the fact that hadacidin is an extracellular product, the possibility of extracellular synthesis was examined by adding [$^{14}$C]formate (5 $\mu$curies, 1 $\mu$mole) to the filtrate of *P. aurantio-violaceum* culture fluid, allowing it to stand for 15 min, and then proceeding with the isolation of hadacidin. A negligible amount of radioactivity was found in the isolated hadacidin, ruling out extracellular synthesis. It also seemed possible that the similar and very high incorporation rates of formate and serine might represent a rapid exchange reaction involving the formyl group of hadacidin rather than net synthesis. This possibility was tested in the following manner: 15 min after the addition of 0.236 $\mu$curie of [$^{14}$C]formate (0.047 $\mu$mole) to the culture flask, 22 mmoles of formic acid or 6 mmoles of ammonium formate was added, and the culture was allowed to shake an additional 15 min. The incorporation (64%) of counts into hadacidin was similar to that of a control flask (59%) to which no chase was added. These experiments rule out the hypothesis that the formyl group of hadacidin is labile. The possibility of a rapid turnover of hadacidin was also examined by adding 10.9 mg of hadacidin labeled in the glycine portion of the molecule (60,000 cpm/mg) to the organism and isolating the hadacidin 24 hr later. In this experiment 100% of the radioactivity was recovered as hadacidin.

[$^{14}$C]*N*-Hydroxyglycine, [carboxyl-$^{14}$C]formylglycine, and [$^{14}$C]glyoxylic acid oxime were compared to [1-$^{14}$C]-glycine as possible hadacidin precursors (Table II). No carrier was added to the radioactive compounds

TABLE II: Relative Incorporation of Glycine, *N*-Hydroxyglycine, Formylglycine, and Glyoxylic Acid Oxime into Hadacidin.

| Compound | Incorporation Time (hr) | Amount Added ($\mu$curies) | Specific Activity ($\mu$curies/mmole) | % Incorporation into Hadacidin |
|---|---|---|---|---|
| [1-$^{14}$C]Glycine | 1 | 0.242 | 3.78 | 26[a] |
| | 3 | 0.242 | 3.78 | 48[a] |
| | 21 | 0.255 | 2.76 | 48 |
| | 48 | 0.215 | 1.96 | 46 |
| [$^{14}$C]*N*-Hydroxy-glycine | 1 | 0.215 | 2.88 | 25 |
| | 3 | 0.261 | 2.88 | 52 |
| | 21 | 0.223 | 3.55 | 64 |
| | 48 | 0.286 | 2.88 | 87 |
| | 72 | 1.23 | 3.55 | 83 |
| | 72 | 0.429 | 3.55 | 85 |
| [Carboxyl-$^{14}$C]formyl-glycine | 1 | 0.125 | 0.299 | 2 |
| | 3 | 0.130 | 0.299 | 3 |
| | 48 | 0.307 | 2.40 | 23 |
| | 48 | 0.591 | 2.40 | 24 |
| [1,2-$^{14}$C]Glyoxylic acid oxime | 3 | 0.105 | 0.91 | 0.6 |
| | 21 | 0.105 | 0.91 | 1.0 |

[a] Average of two experiments.

tested, and the amounts and specific activities were kept as close as possible to ensure comparable results. At 3 hr the formylglycine was incorporated only 3%; this value is to be compared to the 3-hr incorporation values of 52% for *N*-hydroxyglycine and 48% for glycine. Glyoxylic acid oxime was incorporated less than 1% at 3 hr and did not reach higher values for longer time periods. Glycine reaches a maximum incorporation of 48% at 3 hr and levels off at this value, while *N*-hydroxyglycine is incorporated to the extent of 87% at 2 days and maintains this high value at 3 days.

The isotopic dilution experiments (Table III) were designed to show whether *N*-hydroxyglycine, formylglycine, nitroacetic acid, or glyoxylic acid oxime follow glycine in its biosynthetic route to hadacidin. Addition of a compound that is an intermediate in the biosynthetic pathway should dilute out the incorporation of labeled glycine in a given time period. It can be seen that formylglycine showed only a slight dilution effect in an 8-hr incubation period. An equivalent amount of *N*-hydroxyglycine reduced the incorporation of labeled glycine to less than one-third that of the control. During this experiment there was a net synthesis of hadacidin, as noted in the last column of the table. Addition of *N*-hydroxyglycine caused greater than 50% increase in hadacidin production over the control flask. The stimulation of hadacidin synthesis by *N*-hydroxyglycine is reproducible, although the magnitude of the effect varies from culture to culture. In one experiment a 3-fold stimulation of hadacidin synthesis was observed upon addition of *N*-hydroxyglycine for a 3-hr period. None of the other compounds tested in this work, including glycine, showed significant stimulatory or inhibitory effect upon hadacidin biosynthesis. Disodium nitroacetate and glyoxylic acid oxime were also tested as possible intermediates in a 3-hr dilution experiment (Table III). From the incorporation values found, it is apparent that neither nitroacetate nor glyoxylic acid oxime dilute out glycine in its biosynthetic route to form hadacidin.

The location of label in hadacidin formed from the various labeled compounds tested as precursors was determined by a degradation procedure in which the formyl and glycyl portions of hadacidin were separated and assayed for radioactivity. These results are shown in Table IV and demonstrate that the carboxyl group of glycine and *N*-hydroxyglycine are incorporated only into the glycyl portion of hadacidin, while both formate and C-2 of glycine are randomized to a significant degree. In addition, doubly labeled formylglycine was synthesized with $^{14}$C in both the formyl and glycyl portions of the molecule. The synthetic formylglycine was degraded and a ratio of [$^{14}$C]formyl/[$^{14}$C]glycyl of 1.6 was found. This formylglycine (11.05 mg at 105,700 cpm/mg) was added to a culture, and hadacidin was isolated after 3 days of incubation. The hadacidin was degraded and the ratio of [$^{14}$C]formyl/[$^{14}$C]glycyl was found to be 5.1. The total incorporation of $^{14}$C was 23%. The marked change in the ratio of label in the formyl and glycyl portions of the molecule indicate degradation of formylglycine and separate incorporation of formate and glycine into hadacidin.

The source of the oxygen of the *N*-hydroxy group of hadacidin was determined with isotopic oxygen. The

TABLE III: Dilution of Incorporation of Labeled Glycine into Hadacidin by Suspected Hadacidin Precursors.

| Nonradioactive Compound (1.2 mmoles) | [1-$^{14}$C]Glycine Added ($\mu$curies)[a] | Incubation Time (hr) | % Incorporation of Glycine | Hadacidin Synthesized during Incubation (mg) |
|---|---|---|---|---|
| *N*-Hydroxyglycine[b] | 0.47 | 8 | 15 | 61 |
| Control | 0.47 | 8 | 49 | 39 |
| Formylglycine[b] | 0.47 | 8 | 40 | 30 |
| Control | 0.47 | 8 | 49 | 39 |
| Glyoxylic acid oxime[c] | 1.24 | 3 | 50[d] | — |
| Control | 1.24 | 3 | 46 | — |
| Nitroacetic acid[e] | 1.24 | 3 | 51[d] | — |
| Control | 1.24 | 3 | 47 | — |

[a] Specific activity, 5.38 mcuries/mmole. [b] Added 1 hr prior to glycine addition. [c] Added 30 min prior to glycine addition. [d] Average of two experiments. [e] The nitroacetic acid was added in three equal portions at 1-hr intervals starting 30 min prior to glycine addition. The half-life of nitroacetic acid is about 25 min under the experimental conditions.

TABLE IV: Distribution of Label in Hadacidin Synthesized from Different Precursors.

| Source of Label | % of Label in Hadacidin: Glycyl Group | % of Label in Hadacidin: Formyl Group |
|---|---|---|
| [1-$^{14}$C]Glycine | 92[a] | 4[a] |
| [2-$^{14}$C]Glycine | 74[a] | 27[a] |
| [$^{14}$C]Sodium formate | 26[a] | 76[a] |
| DL-[3-$^{14}$C]Serine | 29[a] | 65[a] |
| [$^{14}$C]*N*-Hydroxyglycine | 98 | 0 |
| [Carboxyl-$^{14}$C]formylglycine | 98 | 2 |

[a] Average of two or more degradations.

results of the $^{18}$O experiments are summarized in Table V. In experiment no. 1, a 2-day culture of *P. aurantio-violaceum* was grown in an atmosphere of $^{18}O_2$ for 3 additional days. In experiment no. 2, the organism was grown for 7 days using $H_2{}^{18}O$. Hadacidin was then isolated from both flasks and reduced to formylglycine, thus removing the hydroxylamino oxygen atom. The $^{18}$O atom per cent excess found in the *N*-hydroxy group was calculated in the $^{18}O_2$ experiment to be 1.282, in good agreement with the theoretical value of 1.365. In the $H_2{}^{18}O$ experiment the $^{18}$O atom per cent excess of the *N*-hydroxy group was only 0.206, as compared to values of 1.106 found in the carbonyl and carboxyl oxygens, which are derived from water. In theory no isotope should have appeared in the hydroxylamino oxygen, and the value of 0.206 is well above experimental error. Although we have no explanation as to how this amount of isotope from $H_2{}^{18}O$ entered the hydroxylamino group, we conclude that the data adequately demonstrate that *P. aurantio-violaceum* utilizes atmospheric oxygen in forming the *N*-hydroxy group of hadacidin. The $H_2{}^{18}O$ exchange experiments clearly indicate that neither hadacidin nor formylglycine exchange significantly with $H_2{}^{18}O$ under the experimental conditions.

## Discussion

Dulaney (1963) observed that substrate amounts of glycine added to *P. aurantio-violaceum* cultures did not stimulate hadacidin production and he therefore suggested that glycine is perhaps not an intermediate in hadacidin biosynthesis. Caution is required in the interpretation of negative data of this kind. The failure of valine and cysteine, known precursors of penicillin, to stimulate production of that antibiotic is just one example of the inability of precursors to augment production of an end product of metabolism (Behrens, 1949).

The results reported in this paper demonstrate that [1-$^{14}$C]glycine is in fact very rapidly incorporated into hadacidin. In view of the multiplicity of known metabolic pathways involving glycine, this high incorporation not only indicates that glycine is undoubtedly a hadacidin precursor, but also that under the conditions chosen in our experiments the metabolism of *P. aurantio-violaceum* is directed toward hydroxamate biosynthesis. Formate can likewise be metabolized by several pathways of metabolism in fungi (Foster, 1949), and the high degree of incorporation of formate into hadacidin again indicates the quantitative importance of hydroxamate biosynthesis in *P. aurantio-violaceum*. The equivalence of formate and the $\beta$-carbon of serine in short-time incorporation experiments suggests an extremely active C-1 metabolism in this organism. Our data also demon-

TABLE V: Incorporation of $^{18}O_2$ and $H_2{}^{18}O$ into Hadacidin.[a]

| Expt No. | Source of Oxygen | Compd Analyzed | Atom % Excess Found | Atom % Excess Corrected for Controls | Atom % Excess in Carboxyl and Formyl Groups Calcd[b] | Atom % Excess in Carboxyl and Formyl Groups Found | Calculated Atom % Excess in Hydroxylamino Group Calcd[b] | Calculated Atom % Excess in Hydroxylamino Group Found[c] | Source of Oxygen of Hydroxylamino Group % from $O_2$ | Source of Oxygen of Hydroxylamino Group % from $H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | $O_2$ | 1.365 | 1.365 | — | — | | | | |
| | $^{18}O_2$ | Hadacidin | 0.307 | 0.308(A) | 0 | 0.029 | 1.365 | 1.282(C) | 94 | 6 |
| | | Formylglycine (reduced hadacidin) | 0.029 | 0.029(B) | 0 | 0.029 | | | | |
| 2 | | $H_2O$ | 1.301 | 1.301 | — | — | | | | |
| | $H_2{}^{18}O$ | Hadacidin | 0.877 | 0.881(a) | 1.301 | 1.106 | 0 | 0.206(c) | 84 | 16 |
| | | Formylglycine (reduced hadacidin) | 1.084 | 1.106(b) | 1.301 | 1.106 | | | | |
| Control 1 | | $H_2O$ | 1.316 | — | — | — | | | | |
| | | Hadacidin | 0.006 | — | — | — | | | | |
| Control 2 | | $H_2O$ | 1.316 | — | — | — | | | | |
| | | Formylglycine | 0.021 | — | — | — | | | | |

[a] See the experimental section for conditions of the incorporation experiments and exchange experiments (controls). [b] The theoretical values are based on the assumption that the source of oxygen of the carboxyl and formyl groups is water and that the oxygen of the hydroxylamino group is derived from oxygen gas. If the volume of $^{18}O_2$ (5 l.) that was reduced to water in the $^{18}O_2$ experiment is considered, the theoretical atom % excess of $^{18}O_2$ in the carboxyl and formyl groups would be from 0.01 to 0.05. [c] The equations used to calculate the atom % excess of $^{18}O$ in the hydroxylamino group are $(3B + C/1.12)/4 = A$ and $(3b + c)/4 = a$. The factor 1/1.12 is to correct for the amount of hadacidin synthesized during the 3 days prior to addition of $^{18}O_2$ to the system.

strate that hadacidin is a stable end product of metabolism, there being neither net degradation of hadacidin once released from the cell nor exchange of the formyl group of hadacidin with isotopic formate.

Although our tracer experiments and isotope dilution data indicate that *N*-hydroxyglycine is an intermediate in the conversion of glycine to hadacidin, the possibility that *N*-hydroxyglycine is first enzymically reduced to glycine in the cell has not been positively excluded. We believe that such a conversion is unlikely, however, for two reasons. First, in time periods exceeding 20 hr, the incorporation of *N*-hydroxyglycine into hadacidin exceeds the incorporation of glycine. The 87% incorporation of *N*-hydroxyglycine in a 48-hr period was unsurpassed by any other substrate tested and was almost twice that of [1-$^{14}$C]glycine. This would be expected if pathways of metabolism other than hadacidin synthesis are available to glycine but not to *N*-hydroxyglycine. Second, *N*-hydroxyglycine is the only organic substrate tested that stimulates hadacidin synthesis. Although this effect may be nonspecific, it is suggestive of a precursor relationship. Since neither nitroacetic acid nor glyoxylic acid oxime were incorporated into hadacidin, the possibility of oxidation of glycine to a higher oxidation state followed by reduction to the hydroxylamino acid is unlikely.

The hydroxylamino oxygen atom of hadacidin has been shown by our $^{18}O$ experiments to be derived from atmospheric oxygen, ruling out the possibility that hydration of iminoacetic acid to *N*-hydroxyglycine is a source of the hydroxylamino group. Since our tracer studies have implicated *N*-hydroxyglycine as an intermediate in the conversion of glycine to hadacidin, the $^{18}O$ experiments demonstrate that this conversion represents oxygenation of the amino group of glycine. Baker and Chaykin (1962) have shown that oxygen gas is the source of the oxygen atom of trimethylamine *N*-oxide derived by the enzymic oxidation of trimethylamine. It should be interesting to determine if the mechanism of oxidation of a tertiary amine is related to the oxidation of a primary amino group reported in this paper. Also of interest is the fact that in the course of our $^{18}O_2$ experiments it was observed that the 90–95% oxygen atmosphere employed caused a 2- to 3-fold stimulation of hadacidin synthesis, an effect that was also observed in several preliminary $^{16}O_2$ experiments. It is tempting to speculate that *N*-hydroxylation of glycine is the rate-limiting step in hadacidin synthesis, and that the 4- to 5-fold increase in partial pressure of oxygen is responsible for the increase in the rate of hydroxamate synthesis. However, this effect may merely

be due to a general elevation of the oxidative metabolism of the organism.

Formylglycine is very poorly incorporated into hadacidin, but it may well be that this compound does not rapidly penetrate the cell. This possibility would obviously invalidate any conclusions from our data concerning the metabolic role of intracellular formylglycine. The slow incorporation of formylglycine and the change in ratio of the radioactivity of the formyl to the glycyl group upon incorporation into hadacidin might represent extracellular hydrolysis of the administered formylglycine and subsequent utilization of the formate and glycine. However, the implication of *N*-hydroxyglycine as a hadacidin precursor leads us to believe that formylglycine is not involved in hadacidin synthesis.

Recently, Micetich and MacDonald (1965) have reported that flavacol is an intermediate in the biosynthesis of the cyclic hydroxamic acid, neoaspergillic acid, in *Aspergillus sclerotiorum*. This finding indicates that *N*-oxidation of the heterocyclic nitrogen of the pyrazine ring occurs in the biosynthesis of this hydroxamate and that *N*-hydroxyleucine is not an intermediate. It thus appears possible that cyclic hydroxamates of the aspergillic acid type, in which the hydroxamate group is part of an aromatic ring system, are synthesized by a route different from that of an hydroxamate such as hadacidin, in which an alkyl hydroxylamino group is involved in the hydroxamic acid bond.

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