Initial Steps in the Biosynthesis of Ferrichrome. Incorporation of δ-*N*-Hydroxyornithine and δ-*N*-Acetyl-δ-*N*-hydroxyornithine*

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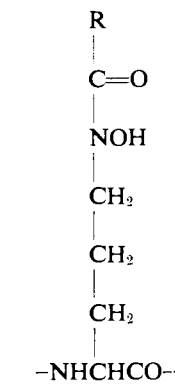
ABSTRACT: δ-*N*-Hydroxyornithine and δ-*N*-acetyl-δ-*N*-hydroxyornithine were investigated as possible precursors of the trihydroxamic acids, ferrichrome and ferrichrome A, in *Ustilago sphaerogena*. δ-*N*-Hydroxyornithine was prepared by hydrolysis of fusarinine. The instability of δ-*N*-hydroxyornithine was circumvented by its conversion to the stable crystalline derivative, 1-hydroxy-3-amino-2-piperidone. δ-*N*-Acetyl-δ-*N*-hydroxyornithine was prepared by acetylation of δ-*N*-hydroxyornithine at pH 4 with acetic anhydride. Approximately 60% of [^{14}C]glycine and L-[^{14}C]ornithine was incorporated into ferrichrome and ferrichrome A in a 48-hr incubation period. D-Ornithine was not incorporated. Degradation of the hydroxamates showed little or no randomization of label except for the conversion of glycine to serine. δ-*N*-Hydroxyornithine, in spite of its instability under our experimental conditions, was incorporated to an extent of 37% into the hydroxamates in a 6-hr period, as compared to 15% for L-ornithine. δ-*N*-Acetyl-δ-*N*-hydroxyornithine was found to be incorporated into ferrichrome, but not into ferrichrome A. Fusarinine and reduced fusarine were not incorporated under the same conditions, indicating that synthesis of the peptide hydroxamates exhibits specificity toward the acyl group of the ornithine-δ-hydroxamate. The experimental results suggest that the biosynthesis of ferrichrome proceeds by oxygenation of ornithine to δ-*N*-hydroxyornithine followed by N acetylation to form δ-*N*-acetyl-δ-*N*-hydroxyornithine. Three molecules of the latter compound form peptide bonds with three molecules of glycine to form ferrichrome.

Evidence has been presented previously that the biosynthesis of hadacidin, *N*-formyl-*N*-hydroxyglycine, proceeds by oxygenation of the amino group of glycine to yield *N*-hydroxyglycine, which is then N formylated to form the hydroxamic acid (Stevens and Emery, 1966). δ-*N*-Hydroxyornithine is one of the most commonly found constituents of naturally occurring hydroxamic acids (Keller-Schierlein *et al.*, 1964). Ferrichrome and ferrichrome A, produced by *Ustilago sphaerogena*, each contain three molecules of this hydroxylamino acid in peptide linkage (Emery and Neilands, 1960, 1961).

The biosynthesis of the ferrichromes has not been previously investigated, and our results with hadacidin suggested to us that δ-*N*-hydroxyornithine might be a precursor. Acetylation of the hydroxylamino group would then yield δ-*N*-acetyl-*N*-hydroxyornithine, the δ-hydroxamic acid derivative of ornithine found in ferrichrome. Alternatively, acylation with *trans*-β-methylglutaconic acid would yield the hydroxamic acid subunit of ferrichrome A.

This paper describes the chemical synthesis of ^{14}C-labeled δ-*N*-hydroxyornithine and δ-*N*-acetyl-δ-*N*-hydroxyornithine, and their incorporation into the ferrichrome compounds by *Ustilago*. The experimental results are in accord with the postulate that the first steps in the biosynthesis of ferrichrome are analogous



ferrichrome, R = CH_3
ferrichrome A, R = $HOOCCH_2C(-CH_3)=CH$

to those of hadacidin biosynthesis, *i.e.*, conversion of an amino group to an hydroxylamino group followed by acylation of the hydroxylamino group to yield the hydroxamic acid. In view of the widespread occurrence of hydroxamic acids in microorganisms, it would appear that these reactions take place in many, if not most, aerobic microorganisms.

Experimental Section

Growth Conditions and Ferrichrome Isolation. U. sphaerogena (ATCC 12421) was grown in a 500-ml culture in 2-l. Fernbach flasks in the iron-deficient medium described by Garibaldi and Neilands (1955). All chemicals were reagent grade and deionized water

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was used. The flasks were incubated at 26° on a reciprocal shaker with a 3.8-cm stroke at 95 strokes/min. The inoculum was 1 ml of a 24-hr culture of the organism grown on the same medium. Where noted, 50-ml aliquots of a 72-hr culture were transferred to 250-ml erlenmeyer flasks. The radioactive substrates were added in a volume of 2 ml and the flasks shaken at 120 strokes/min.

Ferrichrome and ferrichrome A were isolated by the method of Garibaldi and Neilands (1955). Controls showed the extraction procedure to give 85–90% recovery. Ferrichrome A was separated from ferrichrome by paper electrophoresis on Whatman 3MM paper. The buffer was pyridine–acetic acid–water (14:10:930), pH 5.0, and a potential of 38 v/cm was applied for 1.5 hr. The colored bands were eluted from the air-dried paper with water and the extracts were made to a volume of 5 ml. The hydroxamate concentration was determined spectrophotometrically (ferrichrome, λ_{max} 425 mμ (ε 2895); ferrichrome A, λ_{max} 440 mμ (ε 3740)). Further purification was achieved by paper chromatography with 75% pyridine as solvent (ferrichrome, R_F 0.77; ferrichrome A, R_F 0.45). If necessary, a second paper chromatographic purification was performed using butanol–ethanol–water (4:1:5) (upper phase) (ferrichrome, R_F 0.28; ferrichrome A, R_F 0.03), which yielded samples of constant specific activity.

Radioactivity Measurements. Aliquots containing no more than 0.8 mg of ferrichrome or ferrichrome A were plated on aluminum planchets with a diameter of 2 cm and counted in a Nuclear–Chicago Geiger–Müller counter equipped with a micromil window. Samples were counted to a standard deviation of ±2% of the detected counts, except where the samples were essentially unlabeled. All counting data are corrected for background (23 cpm). Paper chromatograms were scanned with a Vanguard 880 strip scanner. All of the counts were found in the orange-colored area of the hydroxamic acids.

Degradation of Ferrichrome and Ferrichrome A. Iron was removed from the ferrichrome and ferrichrome A (Emery and Neilands, 1960), and samples of about 5 mg were reductively hydrolyzed with 48% HI for 16 hr at 110°. After removal of HI by evaporation *in vacuo* over NaOH flakes, the amino acids (glycine, serine, and ornithine) were separated by paper electrophoresis using 3.5% formic acid buffer. The amino acids were eluted from the paper and assayed (Troll and Cannan, 1953). Aliquots were plated for counting as above. The acyl groups of the hydroxamates were removed from the acid hydrolysate by ether extraction. *trans*-β-Methylglutaconic acid was plated directly. Acetic acid was counted in a Technical Measurement Corp. LP-2A liquid phosphor counter.

Preparation of D- *and* L-[^{14}C]*Ornithine.* Uniformly labeled L-[^{14}C]ornithine was prepared by the action of arginase (Worthington) on L-[^{14}C]arginine (Greenstein and Winitz, 1961). D-[5-^{14}C]Ornithine was prepared by allowing a culture of *Escherichia coli* 39A-23R3 to grow in the presence of DL-[5-^{14}C]ornithine (Emery, 1965). After incubation for 22 hr, the concentration of ornithine in the medium dropped to one-half the starting concentration. Both samples of radioactive ornithine were adsorbed on small columns of the hydrogen form of AG 50W-X8 resin (Bio-Rad) and eluted with 3 N NH_4OH. Paper electrophoresis and chromatography showed ornithine to be the only ninhydrin-reactive substance present, and all radioactivity was associated with the ornithine.

Preparation of 1-Hydroxy-3-amino-2-piperidone. Approximately 100 mg of fusarinine (Emery, 1965) was hydrolyzed in 2 ml of 1 N HCl at 80° for 30 min. After extracting four times with ether, the aqueous solution, containing δ-*N*-hydroxyornithine, was taken to dryness *in vacuo* at 35°. The residue was taken up in methanol and taken to dryness three times as above. The residue was dissolved in 3 ml of methanol and kept at 70° for 2 hr under N_2, keeping the volume constant by addition of methanol. The solution was then allowed to concentrate to about 0.5 ml and the product was crystallized by addition of ethanol. Recrystallization from methanol–ethanol gave white needles, mp 211–212° dec.

Anal. Calcd for $C_5H_{10}N_2O_2 \cdot HCl$: C, 36.10; H, 6.65; N, 16.81. Found: C, 35.68; H, 6.85; N, 16.55.

Radioactive 1-hydroxy-3-amino-2-piperidone was prepared by isolation of radioactive fusarinine from a *Fusarium roseum* culture to which 60 μc of DL-[5-^{14}C]-ornithine (4 mc/mmole) were added in three portions between the 4th and 7th days of growth. Fusarinine was isolated on the 8th day. Crystalline 1-hydroxy-3-amino-2-piperidone with a specific activity of 2.3 × 10^6 cpm/mmole was obtained from this fusarinine.

Preparation of δ-N-Acetyl-δ-N-hydroxyornithine. 1-Hydroxy-3-amino-2-piperidone (100 mg) was hydrolyzed in 2.5 ml of 3 N HCl for 12 min at 100°. The solution was taken to dryness at 50° under N_2 and the residue was dissolved in 1 ml of water and adjusted to pH 4.3 with pyridine. Acetic anhydride (80 μl) was added over a period of 40 min, the temperature being kept at 50°. The neutral product was purified by paper electrophoresis at pH 5 (above). The compound was crystallized from methanol–ethyl acetate, mp 192–193° dec.

Anal. Calcd for $C_7H_{14}N_2O_4$: C, 44.25; H, 7.42; N, 14.73. Found: C, 44.29; H, 7.66; N, 14.63.

Results

Ferrichrome Production by U. sphaerogena. Neilands (1957) observed that the formation of microbial iron-chelating substances is dependent upon conditions of iron deficiency. The effect of iron upon the formation of ferrichrome and ferrichrome A by *U. sphaerogena* is shown in Figure 1. Iron at a concentration of 10^{-6} M allows rapid cellular growth, but hydroxamic acid production is severely inhibited. The possibility that even the low level of color produced in the assay may be due to colored iron complexes other than hydroxamic acids has not been ruled out. On the iron-deficient medium, the cells grow relatively slowly and never

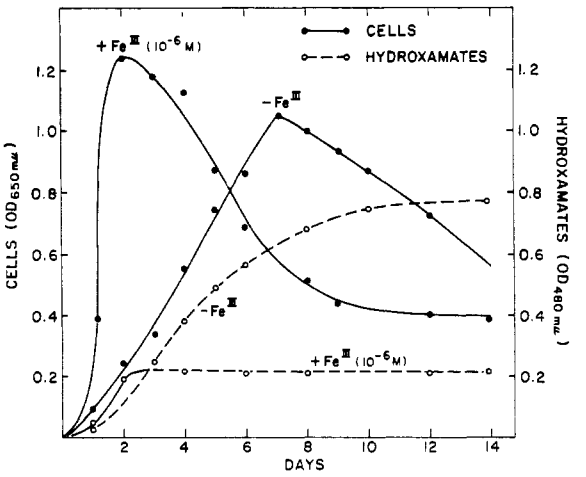


FIGURE 1: Growth of *U. sphaerogena* and hydroxamate production. Cell growth (solid lines) was determined by the optical density at 650 mμ of a 20-fold dilution of the culture. See Experimental Section for growth conditions. Hydroxamates were determined as follows. To 0.5 ml of the clear culture fluid were added 0.05 ml of concentrated HCl and 0.1 ml of 10% $FeCl_3 \cdot 6H_2O$. The volume was made to 5.0 ml and the optical density at 480 mμ determined.

attain the density of those grown on the iron-containing medium. However, excellent hydroxamate formation occurs and continues even after net cellular growth has entirely ceased and is actually declining due to cell lysis. Actual isolation and crystallization of ferrichrome and ferrichrome A showed that the formation of these two compounds is parallel, and no other significant amounts of colored iron chelates are detected in the culture medium. The ferrichromes are stable end products of metabolism, since there is no decrease in their concentrations in the culture fluid even after 3 weeks of incubation. Hydroxamate yields are increased by continued subculturing of the organism in the iron-deficient medium and by conditioning the culture flasks by prior growth of the organism in them. Under these conditions, the rate of ferrichrome or ferrichrome A production in a 72-hr culture is approximately 6 mμmoles/ml per hr, and a maximum concentration of 1 μmole/ml for each compound is not uncommon. Only extracellular hydroxamates were isolated in our experiments. The amount of intracellular hydroxamates is negligible because of the small volume of cells present under these growth conditions.

Incorporation of Glycine and Ornithine. Since glycine occurs in the peptide rings of both ferrichrome and ferrichrome A, the incorporation of this amino acid into the hydroxamates was examined and compared to the incorporation of ornithine into the δ-*N*-hydroxyornithine portion of the molecules. The results in Table I show that in a 48-hr period from 50 to 60% of both of these amino acids was incorporated. This high degree of incorporation is comparable to that found for glycine into hadacidin by *Penicillium aurantiovioleaceum* (Stevens and Emery, 1966), and again demonstrates that under appropriate conditions hydroxamic acid biosynthesis becomes a major pathway of cellular metabolism. This is especially striking when one considers that hydroxamate synthesis continues after net protein synthesis, as measured by cell mass, has stopped entirely. Comparison of the incorporation values for the different substrates in Table I is not strictly justified since, in contrast to our later experiments, the experiments were not performed with the identical culture, and variations in the rate of hydroxamate synthesis both before and after substrate addition may have occurred. However, the similar extent of incorporation of ornithine and glycine suggested that ornithine is a precursor of the *N*-hydroxyornithine found in the hydroxamates.

The results of degradation of the ferrichromes (Table II) indicate that little or no randomization of label had occurred in the three precursors tested, other than the formation of serine from glycine. It is noteworthy that there was no appreciable randomization of label between the two-carbon units of glycine and acetate.

TABLE 1: Relative Incorporation of Glycine, Ornithine, and Acetate into Ferrichrome and Ferrichrome A by *Ustilago*.

	Ferrichrome			Ferrichrome A		
Compd Added[a] (mc/mmole)	Amt Isolated (μmoles)	Sp Act. (cpm/μmole)	% Added Counts	Amt Isolated (μmoles)	Sp Act. (cpm/μmole)	% Added Counts
[1-^{14}C]Glycine (2.5)	346	68,000	38	210	57,000	19
DL-[5-^{14}C]Ornithine (4.0)	341	30,000	33[b]	157	49,000	25[b]
[1-^{14}C]Acetate (1.5)	304	15,400	8	193	28,000	9

[a] Each compound (6.2×10^7 cpm, 100 μc) was added to 500 ml of a 48-hr culture of *U. sphaerogena*. Ferrichrome and ferrichrome A were isolated and crystallized after incubation for 48 additional hr. [b] Based on the incorporation of only one enantiomorph (see Table III).

TABLE II: Distribution of Label in Ferrichrome and Ferrichrome A.[a]

Source of Label	Per Cent of Incorporated Counts						
	Ferrichrome			Ferrichrome A			
	Glycine (3)[b]	Ornithine (3)	Acetate (3)	Glycine (1)	Serine (2)	Ornithine (3)	β-Methyl-glutaconate (3)
[1-^{14}C]Glycine	100	<1	<1	44	57	<1	<1
DL-[5-^{14}C]Ornithine	2	97	<1	2	5	89	5
[1-^{14}C]Acetate	<1	<1	88	<1	<1	<1	94

[a] Ferrichrome and ferrichrome A (Table I) were reductively hydrolyzed and the distribution of total counts among the amino acids and acids determined in the hydrolysate (Experimental Section). [b] Figures in parentheses refer to moles per mole of ferrichrome or ferrichrome A.

The *N*-hydroxyornithine residues of the ferrichromes are all of the L configuration (Emery and Neilands, 1960). In view of the optical inversion of some amino acids upon incorporation into peptides of microbial origin (Meister, 1965), we investigated the optical specificity of ornithine incorporation into the ferrichromes. The results of Table III show that only L-ornithine is incorporated to a significant extent. Since DL-[5-^{14}C]ornithine was used in the remainder of our experiments, it was necessary to show that the D isomer does not adversely affect hydroxamate formation. It was found that D-ornithine at a concentration of 10^{-4} M had no effect upon cell growth or hydroxamate synthesis in *Ustilago*. This concentration of D-ornithine is about 17-fold higher than that present in any of the tracer studies described below.

Isolation and Characterization of 1-Hydroxy-3-amino-2-piperidone. Our primary aim was to obtain evidence that the first step in the biosynthesis of the ferrichromes is the conversion of ornithine to δ-*N*-hydroxyornithine by showing that the latter compound is rapidly incorporated into the peptide hydroxamates. However, initial incorporation experiments with δ-*N*-hydroxyornithine led to ambiguous results, and the difficulty was traced to the decomposition of this hydroxylamino acid. Electrophoretic examination of a solution of *N*-hydroxyornithine that had been allowed to stand for 1 week at 5° showed that 60% of the compound was decomposed into two products: an electrophoretically neutral (pH 5) ninhydrin-positive material, and a substance that gave a yellow reaction with ninhydrin and migrated faster than either ornithine or *N*-hydroxyornithine. The nature of the neutral product has not been investigated further, but it is an oxidation product, probably the oxime of γ-glutamic semialdehyde, since spontaneous oxidation of hydroxylamino acids to oximino acids has been described (Spenser and Ahmad, 1961). δ-*N*-Hydroxyornithine can be almost quantitatively converted to the neutral degradation product by 6-hr exposure of a solution of δ-*N*-hydroxyornithine to air at a pH of 5 or above (Figure 2).

The cationic degradation product of δ-*N*-hydroxyornithine was obtained in crystalline form (Experimental Section), and was identified as 1-hydroxy-3-amino-2-piperidone. Acid hydrolysis (Figure 2) of the compound yielded δ-*N*-hydroxyornithine as the sole product; reductive hydrolysis with HI yielded only ornithine. Titration of the hydrochloride with base showed two ionizable groups with approximate pK_a values of 7.5 and 9, which can be assigned to the amino group and hydroxamic acid group, respectively. The equivalent weight obtained from the titration was found to be 169 (calcd 167). The compound forms a colored complex with Fe(III) with an absorption maximum at 440 mμ.

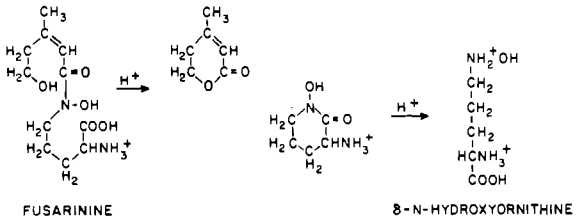


TABLE III: Relative Incorporation of D- and L-Ornithine into Ferrichrome and Ferrichrome A.

Source of Label[a]	Expt	Specific Activity (cpm/μmole)	
		Ferrichrome	Ferrichrome A
D-[5-^{14}C]Ornithine	1	41	24
	2	68	24
L-[^{14}C]Ornithine (U. L.)	1	940	1085
	2	1090	935

[a] The compounds (7.5 μmoles, 2 × 10^6 cpm) were added to 50 ml of a 96-hr culture of *Ustilago* and the ferrichromes isolated 60 hr later.

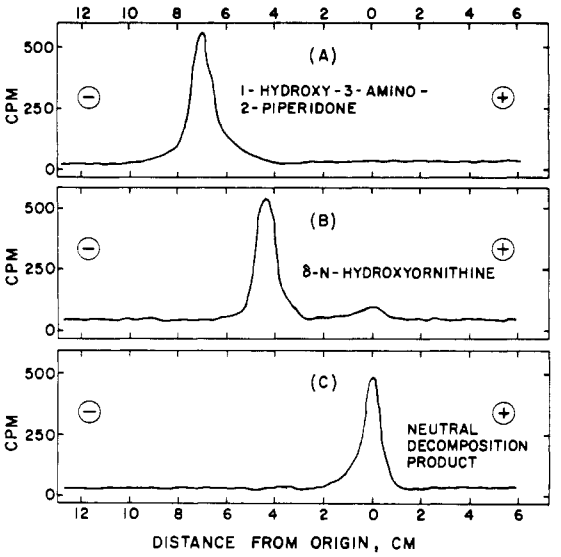


FIGURE 2: Paper electrophoresis of (A) 1-hydroxy-3-amino-2-piperidone, (B) δ-*N*-hydroxyornithine produced by hydrolysis of A for 20 min at 100° in 3 N HCl, and (C) neutral decomposition product formed by aeration of B for 6 hr at pH 6. Electrophoresis was carried out for 0.5 hr in 0.18 N pyridine–acetate buffer, pH 5. The dried papers were scanned for radioactivity with a Vanguard 880 strip scanner. Ornithine moves approximately 6 cm under these conditions.

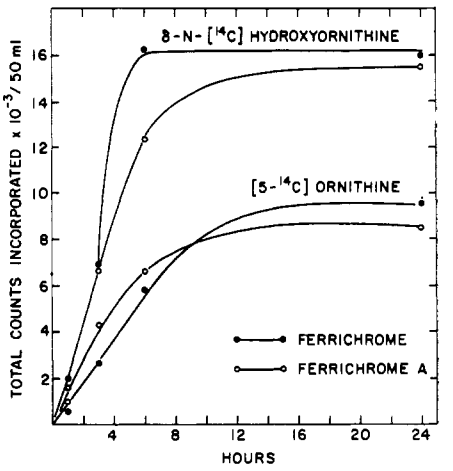


FIGURE 3: Incorporation of [5-^{14}C]ornithine and δ-*N*-[^{14}C]hydroxyornithine into ferrichrome and ferrichrome A by *Ustilago*. Each substrate (30 μmoles, 85,000 cpm) was added to 50 ml of culture and shaken at 26°. Ferrichrome and ferrichrome A were purified from a 5-ml aliquot at the times indicated and the radioactivity was determined.

We previously ascribed the acid instability of fusarinine to the cyclization of the acyl portion of the molecule, *cis*-5-hydroxy-3-methylpent-2-enoic acid, to form the lactone (Emery, 1965). It now appears that the acid instability of fusarinine may also in part be due to cyclization of the hydroxylamino portion of the molecule.

Incorporation of δ-N-Hydroxyornithine into Ferrichrome. Radioactive δ-*N*-hydroxyornithine was obtained by acid hydrolysis of ^{14}C-labeled 1-hydroxy-3-amino-2-piperidone and neutralization of the hydrolysate immediately prior to addition to the culture (Figure 2). The relative incorporation of δ-*N*-hydroxyornithine and ornithine into ferrichrome and ferrichrome A is shown in Figure 3. Incorporation of the hydroxylamino acid reaches a maximum 6–8 hr after addition to the culture. At all time periods examined, the per cent incorporation of *N*-hydroxyornithine exceeded that of ornithine, and the specific activities of the ferrichromes isolated were higher in the case of the hydroxylamino acid (Table IV). It is important to note that these experiments were performed with the identical culture of the organism to preclude cellular variation in the rate of hydroxamate synthesis.

In view of the instability of δ-*N*-hydroxyornithine discussed above, we were concerned that our results might in fact be due to the neutral degradation product rather than the hydroxylamino acid itself. Table IV shows 6-hr incorporation data for the neutral decomposition product as well as 1-hydroxy-3-amino-2-piperidone. The low degree of incorporation of both of these substances enables us to conclude that decomposition of *N*-hydroxyornithine leads to the formation of compounds that are inert with respect to hydroxamate synthesis.

The possibility that *N*-hydroxyornithine was reduced by the cells to ornithine, which was subsequently incorporated into the ferrichromes, was also considered. Barring permeability effects, the greater extent of incorporation of the hydroxylamino acid would argue against this possibility. However, efforts were also made to isolate radioactive cellular ornithine after incubation of the cells for 6 hr with the hydroxylamino acid. These efforts were unsuccessful, although radioactive cellular ornithine could be isolated after incubation of the cells with [5-^{14}C]ornithine. The radioactive ferrichrome and ferrichrome A isolated from experiments using *N*-hydroxyornithine as precursor were reductively hydrolyzed, and all of the incorporated counts were found in the ornithine in the hydrolysate.

Incorporation of δ-N-Acetyl-δ-N-hydroxyornithine into Ferrichrome. δ-*N*-[1-^{14}C]Acetyl-δ-*N*-hydroxyornithine was prepared by acetylation of δ-*N*-hydroxyornithine with acetic anhydride (Experimental Section). This acetylated derivative is the hydroxamic acid subunit of ferrichrome, but not ferrichrome A, and our working hypothesis was that this compound should be incorporated only into ferrichrome if formation of the hydroxamate group does in fact precede the synthesis of the peptide. The data of Table V show that δ-*N*-acetyl-δ-*N*-hydroxyornithine is indeed incorporated into ferrichrome and not into ferrichrome A. δ-*N*-([1-^{14}C]acetyl)ornithine, prepared by the method of Neuberger and Sanger (1943), was tested as a control

TABLE IV: Incorporation of δ-*N*-Hydroxyornithine into the Ferrichromes.

Source of Label[a]	Ferrichrome			Ferrichrome A		
	Recov (μmoles)	Sp Act. (cpm/μmole)	% Counts Incorp	Recov (μmoles)	Sp Act. (cpm/μmole)	% Counts Incorp
[^{14}C]-δ-*N*-Hydroxyornithine	21.8	750	19.2	21.9	560	14.4
DL-[5-^{14}C]Ornithine[b]	20.0	295	7.0	22.2	302	7.9
[^{14}C]-1-Hydroxy-3-amino-2-piperidone	27.0	19	0.6	13.1	12	0.2
[^{14}C]Neutral decomposition product	23.4	24	0.7	14.8	22	0.4

[a] The labeled compounds (30 μmoles, 8.5 × 10^3 cpm) were added to 50 ml of a 72-hr *Ustilago* culture. The hydroxamates were isolated after 6 hr of incubation. [b] L-Ornithine (8.5 × 10^3 cpm, 30 μmoles) containing 0.3 μmole of D-ornithine (8.5 × 10^3 cpm) was added. Per cent incorporation based on L isomer only.

TABLE V: Incorporation of δ-*N*-Hydroxyornithine Derivatives into the Ferrichromes.

Expt	Source of Label[a]	Incubn Time (hr)	Ferrichrome Recov (μmoles)	Ferrichrome Sp Act. (cpm/μmole)	Ferrichrome % Counts Incorp	Ferrichrome A Recov (μmoles)	Ferrichrome A Sp Act. (cpm/μmole)	Ferrichrome A % Counts Incorp
1	δ-*N*-[1-^{14}C]Acetyl-δ-*N*-hydroxyornithine	6	21.2	274	6.8	19.1	49	1.1
	[5-^{14}C]Ornithine (control)[b]	6	17.6	342	7.1	18.8	289	6.4
	δ-*N*-([1-^{14}C]Acetyl)ornithine	6	18.4	12	0.5	18.6	20	0.9
2	δ-*N*-[1-^{14}C]Acetyl-*N*-hydroxyornithine	24	21.6	1270	32.3	17.3	26	0.5
3	[^{14}C]Fusarinine	24	33.1	30	1.1	17.4	29	0.6
	[^{14}C]Fusarinine-H_2	24	30.8	16	0.8	16.5	15	0.4
4	[1-^{14}C]Acetate (control)[c]	24	31.9	220	4.0	25.3	730	10.6

[a] The labeled compounds (30 μmoles, 8.5 × 10^3 cpm) were added to 50 ml of a 72-hr *Ustilago* culture. [b] L-Ornithine (8.5 × 10^3 cpm, 30 μmoles) containing 0.3 μmole of D-ornithine (8.5 × 10^3 cpm) was added. [c] Thirty micromoles (17.4 × 10^3 cpm) was added.

and found not to be incorporated. The incorporation of *N*-acetyl-*N*-hydroxyornithine into ferrichrome, but not ferrichrome A, rules out the possibility that the compound was first degraded to yield acetate, which was subsequently incorporated. A number of precursor experiments have been performed with acetate, and the results invariably demonstrate that the specific activity of the isolated ferrichrome A is greater than that of ferrichrome. Typical data are included in Table V. In addition, δ-*N*-acetyl-δ-*N*-hydroxyornithine labeled in the amino acid portion of the molecule was prepared by acetylation of [^{14}C]*N*-hydroxyornithine and tested in the same manner. The isolated ferrichrome had a specific activity of 656 cpm/μmole, compared to 3 cpm/μmole for ferrichrome A. Although the conditions of this experiment were somewhat different from those presented in Table V, the results agree in showing that the acetylated derivative is exclusively a precursor of ferrichrome.

The structural similarity of fusarinine to the hydroxamic acid subunit of ferrichrome A led us to examine the incorporation of [^{14}C]fusarinine into hydroxamates of *Ustilago*. Reduced fusarinine, δ-*N*-(5-hydroxy-3-methylpentanoyl)-δ-*N*-hydroxyornithine, produced by catalytic hydrogenation of fusarinine, was also tested. The data of Table V show that neither of these compounds was incorporated to a significant degree.

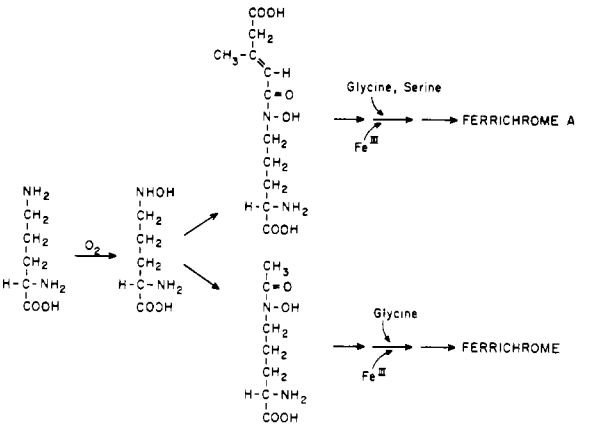
Discussion

The instability of δ-*N*-hydroxyornithine frustrated our initial precursor studies. At pH values near or above the p*K* of the hydroxylamino group, which is about 5, *N*-hydroxyornithine is oxidatively decomposed. Between pH 1 and 4, a cyclization occurs to yield varying amounts of 1-hydroxy-3-amino-2-piperidone. Fortunately, the piperidone is a stable crystalline compound which can be quantitatively converted to the hydroxylamino acid by acid hydrolysis, and its availability allowed us to carry out precursor studies. Other workers have also reported the formation of a compound having the properties of 1-hydroxy-3-amino-2-piperi-

done during structural work on the naturally occurring hydroxamic acid, albomycin (Turková *et al.*, 1962), but the compound was not available in sufficient quantities for crystallization and characterization. The homologous cyclic hydroxamic acid derivative of ε-*N*-hydroxylysine is a constituent of mycobactin (Snow, 1954).

Rapid hydroxamate biosynthesis by *Ustilago* requires vigorous aeration, and the pH of the culture medium falls to about 5. These are precisely the conditions favoring decomposition of δ-*N*-hydroxyornithine; however, we repeatedly observed incorporation of this compound into the ferrichromes in excess of 35% in 6-hr periods, and in one case over 50% incorporation was observed. The rate and extent of *N*-hydroxyornithine incorporation was twice that of ornithine. In view of the instability of the hydroxylamino acid under the conditions of our experiments, it is not surprising that its incorporation was not quantitative, but leveled off after a relatively short time of 6 hr. Since our control studies showed complete decomposition of the compound under similar conditions, it is obvious that penetration into the cells and stabilization by hydroxamate formation must be a very rapid process. The leveling off of ornithine incorporation must undoubtedly be ascribed to the removal of ornithine from hydroxamate synthesis by other pathways of metabolism. Radioactive cellular arginine and glutamic acid were found after incubation of *Ustilago* with [5-^{14}C]ornithine.

Rapid and efficient incorporation of a substrate into a product is not sufficient proof that the compound in question is an intermediate on the biosynthetic pathway (Davis, 1955). Such evidence does, however, indicate the presence of an active metabolic pathway interrelating substrate and product. Our results rule out reduction of *N*-hydroxyornithine to ornithine, and we feel that it is logical to conclude that *N*-hydroxyornithine is an obligatory intermediate in ferrichrome biosynthesis. The failure of efforts to detect *N*-hydroxyornithine in *Ustilago* cells during active hydroxamate synthesis is probably due to the short half-life of the hydroxylamino acid *in vivo*. This is not surprising in view of the high reactivity of the hydroxylamino group. Protection of the hydroxylamino group by *N*-acylation to form a stable hydroxamate therefore seemed to us to be a logical reaction in the biosynthetic sequence. Accordingly, δ-*N*-acetyl-*N*-hydroxyornithine was synthesized and tested as a ferrichrome precursor. The rate of incorporation of this compound was less than that of *N*-hydroxyornithine, but this is probably due to a permeability effect since in a 24-hr period δ-*N*-acetyl-*N*-hydroxyornithine was found to be the most efficient precursor of ferrichrome tested. The incorporation of this compound into ferrichrome and not ferrichrome A is indicative that it is a true intermediate and definitely rules out prior breakdown to acetate and *N*-hydroxyornithine, which would then lead to the formation of radioactive ferrichrome A. We interpret our findings to demonstrate that the pathway of ferrichrome and ferrichrome A biosynthesis in *Ustilago* is



By analogy to our results concerning the biosynthesis of hadacidin, we assume that the oxidation of the δ-amino group of ornithine to a hydroxylamino group utilizes molecular oxygen as substrate.

The acyl groups of all known hydroxamic acids containing δ-*N*-hydroxyornithine are mevalonic acid analogs, or acetate itself, for which the coenzyme A derivatives are either known or can be surmised. It seems likely, therefore, that the acylation of the hydroxylamino group of *N*-hydroxyornithine may well involve coenzyme A. The acyl group of ferrichrome A, *trans*-β-methylglutaconic acid, is known as its CoA derivative (Hilz *et al.*, 1958). The formation of a given hydroxamic acid of the ferrichrome type exhibits specificity not only with respect to the number and sequence of amino acid residues, but also with respect to the acyl groups of the hydroxamic acid functions. Hydroxamates of the ferrichrome type have not been found with mixed acyl functions, *e.g.*, acetate and methylglutaconate, in the same molecule. Since the results of this paper indicate that the acylation of the hydroxylamino group occurs prior to peptide bond formation, we can conclude that for the ferrichrome compounds peptide synthesis is directed by the particular acyl group of the *N*-acyl-*N*-hydroxyornithine. δ-*N*-Acetyl-δ-*N*-hydroxyornithine can form peptide bonds only with itself or glycine to form ferrichrome. The organism acts upon each acyl derivative of *N*-hydroxyornithine as a unique amino acid. Our efforts to trick *Ustilago* into producing new hydroxamic acids containing fusarinine, or reduced fusarinine, were unsuccessful, although fusarinine itself is a subunit of ferrirhodin, produced by *Penicillium versicolor* (Keller-Schierlein, 1963), again demonstrating the specificity of the peptide-synthesizing system.

The *in vivo* oxygenation of amino groups to hydroxylamino groups and the acylation of the latter to form hydroxamic acids are novel biochemical reactions, but by no means restricted to ornithine. Hydroxamic acids involving the *N*-hydroxy derivatives of alanine, tyrosine, glycine, lysine, leucine, and isoleucine are also known (Keller-Schierlein *et al.*, 1964). The enzymes responsible for these reactions are currently being sought in our laboratory.

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Characterization of $\alpha^{23GluNH_2}$ in Hemoglobin Memphis. Hemoglobin Memphis/S, a New Variant of Molecular Disease*

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ABSTRACT: A previously unreported mutant α chain, $\alpha^{23GluNH_2}$, was found associated with β^A as hemoglobin Memphis, with β^{6Val} as Hb Memphis/S, or with β^{6Lys} as Hb Memphis/C. The two hemoglobins, Hb Memphis/S and Hb S, found together cause a new, clinically mild variant of sickle cell anemia. The hemoglobins were characterized by electrophoresis, hybridization, separation of the α and β chains by countercurrent distribution, tryptic and chymotryptic digestion, peptide maps, amino acid analysis, whole blood viscosity, and minimal hemoglobin gelling concentration. The mutation is a substitution of glutamine for glutamic acid at residue 23 in the corner between the A- and B-helical regions of the α chain. The loss of a negative charge in this region may alter the configuration of the α chain.

The molecular behavior of the mutant α-chain combination with β^S differs from that of α^A with β^S. The viscosity of the blood, gelling of the hemoglobin, and the clinical history of the individuals with Hb Memphis/S and Hb S deviate from the findings in a sickle cell anemia (Hb S) individual without the α-chain variant. Thus, the sickling phenomenon may be related to polypeptide chain interaction.

The characterization of a mutation in an abnormal human hemoglobin provides additional confirmation of the genetic code (Beale and Lehmann, 1965) and a means of correlating a change in primary structure of a protein with its tertiary configuration and any resulting change in the molecular behavior. Herein is reported a previously undescribed mutation in the α chain of hemoglobin where glutamine is substituted for glutamic acid at residue 23 ($\alpha^{23GluNH_2} = \alpha^{Mem}$). This abnormal α chain is found associated with another abnormal chain, β^{6Val} (β^S). In this individual no normal β chains are present; therefore, the molecular criterion for identification of sickle cell anemia is met. However, the alleviation of the classical symptoms of sickle cell anemia and the behavior of cells and hemoglobin under low-oxygen tension indicate that the hemoglobin $\alpha_2^{Mem}\beta_2^S$ does not follow the same molecular behavior as $\alpha_2^A\beta_2^S$. This then may be an indication that interaction between α and β chains

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