hydrochloric acid gave silky needles of an acid, m.p. 173°, which was identified as the addition product[6] of HCl with ethyl dicyanoacetate by direct comparison with an authentic sample.[21]

Thus, the original compound was the dihydrated ferrous salt of ethyl dicyanoacetate. Similarly, the dihydrate of the ferrous salt of methyl dicyanoacetate resulted when the reaction was carried out as described above with methanol as solvent.

Reaction of Mercury Iron Tetracarbonyl with Dichlorodiphenylmethane.—Mercury iron tetracarbonyl (3.68 g., 0.01 mole) and dichlorodiphenylmethane (2.37 g., 0.01 mole) were refluxed in 50 ml. of benzene for 0.5 hr. The resulting green solution was filtered to obtain 3.57 g. of light green insoluble residue. The residue contained a water-soluble fraction which was identified as ferrous chloride by qualitative tests, and a water-insoluble fraction which was shown to be $(ClHg)_2Fe(CO)_4$ by comparison of its infrared spectrum with a spectrum of authentic material, and by thermally decomposing the compound to ferrous chloride, mercury and the theoretical amount of carbon monoxide. The original green filtrate was evaporated to dryness and the residue washed with hexane to produce 1.11 g. (0.0033 mole) of tetraphenylethylene, identified by comparison with an authentic sample.

A separate experiment was carried out as above and the liberated carbon monoxide was collected. A total of 0.018 mole of carbon monoxide was evolved. The reaction is described by

$$2HgFe(CO)_4 + 2(C_6H_5)_2CCl_2 \longrightarrow (ClHg)_2Fe(CO)_4 + FeCl_2 + 4CO + (C_6H_5)_2C{=}C(C_6H_5)_2$$

Reaction of Other Metal Carbonyls with Dichlorodiphenylmethane.—In each case the metal carbonyl was refluxed with dichlorodiphenylmethane in benzene for 0.5–3 hr. If any reaction had occurred, as evidenced by color changes or formation of a precipitate, the solution was filtered and the filtrate searched for organic products.

(21) Kindly supplied by Dr. W. J. Middleton.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

Structure of the Ferrichrome Compounds[1,2]

BY THOMAS EMERY AND J. B. NEILANDS

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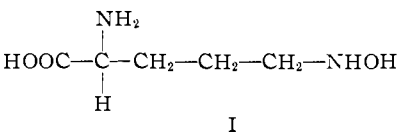
The hydroxyamino moiety of the ferrichrome compounds has been characterized as L-2-amino-5-hydroxyaminovaleric acid (δ-N-hydroxy-L-ornithine). Both ferrichrome and ferrichrome A contain three residues of this new amino acid which together with three residues of glycine (ferrichrome) and one residue of glycine plus two residues of serine (ferrichrome A) are contained within a cyclic hexapeptide. Tentative total structures for the ferrichrome compounds have been proposed.

In previous communications, the ferrichrome compounds were described as naturally-occurring *ferric hydroxamates*[3] and the acyl moieties[4] of the hydroxamate functions were shown to be three residues each of acetic and *trans*-β-methylglutaconic acids for ferrichrome and ferrichrome A, respectively. The present report is concerned with the hydroxyamino functions and, in addition, tentative structures of the entire compounds are proposed which accommodate all of the characterization data including the elementary analyses (Fig. 1).

In the structure illustrated in Fig. 1, the three hydroxyamino functions are shown to be provided by three residues of 2-amino-5-hydroxyaminovaleric acid (δ-N-hydroxyornithine, I). The properties of this new amino acid closely parallel those of the next higher homolog which was isolated from mycobactin in an elegant investigation by Snow.[5] Although substance I has not been obtained from the ferrichrome compounds in analyzable quantities, the existence and structure of this product are strongly inferred from the following evidence.



I

Vigorous acid hydrolysis of the ferrichromes always yielded variable amounts of ornithine, usually 0.3–0.6 mole, in addition to glycine (ferrichrome) and glycine plus serine (ferrichrome A). Quantitative analysis of the hydrolysate of *iron-free* ferrichrome revealed the presence of three moles of glycine; the corresponding values for ferrichrome A were one mole of glycine and two moles of serine. No ornithine was found in hydrolysates from iron-free samples of either compound. However, three moles of I was now present. Apparently I is completely destroyed on acid hydrolysis in the presence of iron and gives rise to variable amounts of ornithine. At *p*H 4.9 I displayed a cationic mobility on paper electrophoresis which was intermediate between that of the neutral amino acids and ornithine. It was stained blue with ninhydrin and gave an immediate red color with the tetrazolium spray employed by Snow[5] for detection of the hydroxyamino grouping. Substance I was eluted from paper electrophoretograms and reduced with H_2 and PtO_2 to an amino acid which could be identified as ornithine. Alternatively, direct reductive hydrolysis of the iron-free ferrichromes with HI yielded three moles of L-ornithine. The configuration of the latter was established by quantitative growth tests with *E. coli* 160-37[6] which in turn indicated the asymmetric form of the parent hydroxyamino acid.

The amino acid sequence of ferrichrome shown in Fig. 1 is ⌐*Gly-Gly-Orn(NHOH)-Orn(NHOH)-Gly-Orn(NHOH)*¬. *The amino acid sequence is tentative and is based on the examination of molecular models.* The three hydroxamic acid functions must be made to satisfy the six octahedrally directed

(1) Abstracted from the doctoral dissertation of Thomas Emery, University of California, Berkeley, 1960.

(2) This research was sustained by a grant from the Office of Naval Research.

(3) T. Emery and J. B. Neilands, *Nature*, **184**, 1632 (1959).

(4) T. Emery and J. B. Neilands, *J. Am. Chem. Soc.*, **82**, 3658 (1960).

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valences of the ferric ion. The arrangement shown was found to best meet this requirement, and the result is a highly symmetrical molecule with the iron centrally located (but not coplanar) with respect to the cyclic peptide ring. The absence of end groups[7] demands the cyclic structure. Also, the enhanced stability of the ferric ion in ferrichrome (as contrasted to simple ferric trihydroxamates) is further evidence that the three hydroxamate functions are all attached to the same molecule.

The proposed structure for ferrichrome A is analogous to that for ferrichrome except that the acyl moieties are three residues of *trans*-β-methylglutaconic acid in which the double bond is α-β to the hydroxamate linkage.[8] In addition to three residues of I, ferrichrome A contains a residue of glycine and two residues of serine. The configuration of the latter amino acid has not been determined.

Ferrichrome A is thus a hexapeptide with three alkyl hydroxyamino side chains each of which is acylated by a residue of an unsaturated dicarboxylic acid. At neutral *p*H the ionic paper electrophoretic mobility of intact ferrichrome A is the same as that of the iron-free compound.[8] This fact, taken in conjunction with the lack of end groups,[9] the presence of three different types of amino acid residues and the enhanced ligand activity[3] is conclusive evidence that the hexapeptide is cyclic–with the iron and all three hydroxamate groupings bonded to the same molecule. The amino acid sequence has not yet been determined.

Compound I is the unique essential structural unit of the ferrichromes, and it therefore appeared desirable to attempt a chemical synthesis of this amino acid. The preparation of I from glutamic acid *via* the 5-γ-bromopropylhydantoin of Gaudry[10] has recently been achieved, and this work will be described in a subsequent publication[11] dealing with the synthesis of ω-N-hydroxyamino acids.

Experimental[12]

Neutral Amino Acid Content of the Ferrichromes.—Exactly 0.5 ml. of a solution containing 8.59 mg. of iron-free ferrichrome[4,7] or 12.50 mg. of iron-free ferrichrome A[4,9] (12.5 μmoles) was hydrolyzed at 110° for 24 hr. in 6 *N* HCl in a sealed tube. After removal of excess acid *in vacuo* over solid NaOH the residue was dissolved in 1 ml. of water and neutralized with $NaHCO_3$. An additional 25 mg. of $NaHCO_3$ was added and the solution was then treated with 2 ml. of a 2% (v./v.) ethanolic solution of 2,4-dinitrofluorobenzene (FDNB). The solution was shaken for 90 minutes at room temperature and the solvent then removed under reduced pressure. The FDNB derivatives of the amino acids were analyzed by two dimensional paper chromatography in the usual way.[13,14]

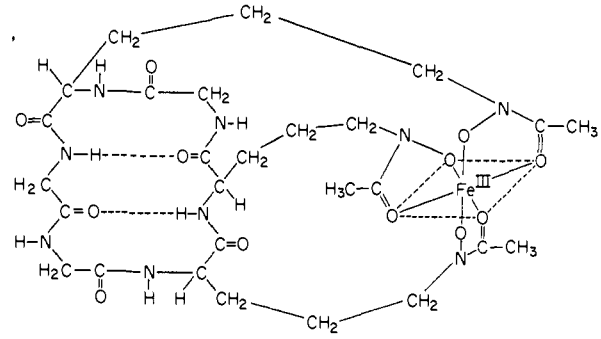

Fig. 1.—Structure proposed for ferrichrome: in the case of ferrichrome A, the acyl moiety is three residues of *trans*-β-methylglutaconic acid[4] while the peptide moiety contains two residues of serine, one residue of glycine and three residues of L-2-amino-5-hydroxyaminovaleric acid (δ-N-hydroxy-L-ornithine, I).

Detection of δ-N-Hydroxyornithine (I).—To 1 ml. of a solution containing approximately 10 mg. of iron-free ferrichrome[4] (or iron-free ferrichrome A[4]) was added 1 ml. of 12 *N* HCl. The solution was heated in a sealed tube for 24 hr. at 110°. The excess acid was removed over NaOH *in vacuo*. The residue was dissolved in 0.1 ml. of water and 0.01 ml. aliquots were analyzed by electrophoresis on Whatman #1 paper. The buffer was pyridine:acetic acid:citric acid:water, 40:30:12:930, *p*H 4.9 and a potential of 35 volts/cm. was applied for 1 hr.

	Moles amino acid/mole ferrichrome compound		
	Glycine		Serine
Ferrichrome	2.9	2.9	nil
Ferrichrome A	1.0	1.2	1.6 2.1

The dried paper sprayed with ninhydrin showed two intense blue spots. The first, near the origin, was glycine (ferrichrome) or glycine + serine (ferrichrome A). The second spot, *i.e.* I, had a cathodic migration of about 11 cm. This second spot gave a red color when sprayed with tetrazolium reagent.[5]

From the behavior of I on paper electrophoresis it is apparent that the pK_a' of the hydroxylammonium ion must be close to 5.0. This is in good agreement with the value of 5.2 reported by Snow[5] for the lysine homolog and is probably too low for an α-hydroxyamino acid.[15] A reference compound, believed to be 2-hydroxyamino-3-methylbutyric acid,[15] was obtained from Dr. K. M. Mann of Upjohn Company. This specimen, although actively reducing toward tetrazolium, gave only a very faint ninhydrin reaction.

Reduction of I to Ornithine (a) Catalytic.—Two aliquots of the above hydrolysate were subjected to paper electrophoresis in duplicate side by side application. The position of the spots for one aliquot was determined by ninhydrin spray. The desired position on the other aliquot then was cut from the paper and placed in a test tube with two ml. of water to which had been added one drop of concd. HCl. The tube was heated to 80° for 5 minutes and the paper then removed and discarded. About 10 mg. of PtO_2 was added and a slow stream of hydrogen was passed through the mixture for 1 hr. The catalyst was removed by centrifugation and the supernatant solution concentrated almost to dryness on a steam-bath. The entire sample was analyzed by paper electrophoresis, as described above. A single spot was found with a cathodic migration of about 16 cm., *i.e.*, the same as that for authentic ornithine.

(b) With HI.—Exactly 1.0 ml. of a solution containing 2.48 mg. (3.61 μmoles) of iron-free ferrichrome was placed in a test tube and the solution evaporated to dryness on a steam-bath. After the addition of 0.5 ml. of 50% HI the tube was sealed and heated at 110° for 24 hr. The tube was opened and the contents taken to dryness *in vacuo* over NaOH. The residue was dissolved in 0.5 ml. of water, 0.2 ml. of 0.02 *N* sodium thiosulfate was added and the entire

(7) J. B. Neilands, *J. Am. Chem. Soc.*, **74**, 4846 (1952).

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(12) Microanalysis by Chemistry Department, University of California, Berkeley.

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(14) H. Fraenkel-Conrat and B. Singer, *Arch. Biochem. Biophys.*, **60**, 64 (1956).

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sample diluted with water to 25 ml. in a volumetric flask. Ornithine was determined by the method of Chinard.[16] Thiosulfate and glycine were found not to interfere with the analysis. Values of 2.9, 3.1 and 2.9 moles ornithine/mole ferrichrome were found in triplicate analyses.

Analysis for L-Ornithine.—The assay was conducted with *E. coli* mutant 160–37 which was kindly supplied by H. J. Vogel.[6] Glycine and D-ornithine were found not to interfere. Triplicate analyses of the HI hydrolysate described above gave 2.8, 3.2 and 2.8 moles L-ornithine/mole ferrichrome.

Syntheses of I.—The preparation of the ω-N-hydroxy derivatives of DL-ornithine and DL-lysine will be the subject of a succeeding communication.[11] The following procedure relates to the preparation of DL-compound I in sufficient quantities for comparison with the natural product.

A solution containing 500 mg. (2.26 mmoles) of DL-5-γ-bromopropylhydantoin,[10] 250 mg. (3.62 mmoles) of $NaNO_2$ and 250 mg. of urea in 5 ml. of dimethylformamide was agitated with a magnetic stirrer for 2 hr. at room temperature as described by Kornblum, *et al.*[17] The solvent was then removed and the residue extracted with acetone. The acetone extracts were filtered to yield a clear yellow solution. The acetone was evaporated and the residue dissolved in 10 ml. of ethanol. After addition of 3 ml. of water and 500 mg. of NH_4Cl, the solution was heated to near boiling and 500 mg. of zinc dust added in portions over a period of several minutes. The solution was then filtered, acidified with dilute HCl and evaporated to dryness. The residue was dissolved in 2–3 ml. of water and applied to a 1 × 5 cm. column of Dowex 50H. The column was washed with water and the tetrazolium positive material eluted with 0.2 *M* NH_4OH. The tetrazolium-positive fractions were pooled, acidified with dilute HCl and evaporated to dryness. The residue was dissolved in 1 ml. of 6 *N* HCl and heated in an autoclave at 135° for 4 hr. After removal of excess HCl, this preparation on electrophoretic analysis (see above) showed the presence of approximately equal portions of ninhydrin positive materials with the mobility characteristics of neutral amino acid(s), compound I and ornithine.

The entire preparation was placed on a 1.5 × 50 cm. column of 200–400 mesh Dowex 50.H × 4 equilibrated with 2.5 *N* HCl. The column was operated at a flow rate of one fraction (4 ml.) per 12 min. with 2.5 *N* HCl as developing solvent. The neutral amino acid(s) as well as an unidentified tetrazolium-reducing peak emerged within the first 50 fractions. Fractions 50–60 gave a positive spot test on paper with tetrazolium. These fractions were pooled, evaporated to dryness and the total residue applied in a narrow band across the entire 15 cm. width of a sheet of Whatman 3 MM filter paper. A spot of authentic ornithine was placed on the origin at one side of the paper. The buffer and operating conditions were the same as those used above. After approximately 1 hr. the paper was air dried and a strip cut off along the edge bearing the authentic ornithine spot. The ninhydrin spray revealed the presence of only two components, the authentic ornithine (15.7 cm.) and compound I (11.1 cm.). A second strip was sprayed with ninhydrin and this showed only a band at 11.1 cm. Finally, a third strip sprayed with tetrazolium reagent gave one band at the 11.1 cm. position.

The 11.1 cm. band from the remainder of the paper was eluted and reduced with HI as was done with the natural product (see above). Paper electrophoretic analysis of the reduced preparation revealed the presence of ornithine as the sole amino acid.

Synthetic and natural I were indistinguishable by ionic mobility on paper and by their behavior toward the ninhydrin and tetrazolium reagents. In the experiment described above, the ionic mobility ratio of synthetic I/ornithine was 11.1/15.7 = 0.708. In a separate but similar analysis the corresponding ratio for natural I/ornithine was 10.1/14.3 = 0.706.

Elementary Analyses. (a) **Ferrichrome.**—Calcd. for $C_{27}H_{42}N_9O_{12}Fe$: C, 43.79; H, 5.72; N, 17.02; Fe, 7.54. Found: C, 44.02; H, 5.90; N, 16.55; Fe, 7.54.

(b) **Ferrichrome A.**—Calcd. for $C_{41}H_{58}N_9O_{20}Fe\cdot 4H_2O$: C, 43.78; H, 5.91; N, 11.21. Found: C, 43.62; H, 5.80; N, 11.18.

A thrice recrystallized sample was dried at 100° under reduced pressure over P_2O_5 for 24 hr. The specimen was then allowed to reach constant weight under atmospheric conditions. The dry sample, 26.5 mg. (25.17 μmoles) reached constant weight at 28.3 mg. The water absorbed, 1.8 mg. (100 μmoles) was 99.3%.

Anal. Calcd. for $C_{41}H_{58}N_9O_{20}Fe$: Fe, 5.3. Found: Fe, 5.3.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA, IOWA CITY, IOWA]

Ionization-linked Changes in Protein Conformation. I. Theory

BY CHARLES TANFORD*

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If a protein molecule can exist in two conformations, α and β, and if the equilibrium constant (β)/(α) depends on *p*H, then a necessary consequence of the laws of thermodynamics is that at least one titratable group has a different pK in the two conformations. The objective of this paper is to give exact equations for the relation between the *p*H-dependence of (β)/(α) and the course of titration of the anomalous titratable groups, in a form suitable primarily in situations where electrostatic interaction does not play an important role.

If a protein molecule in solution can exist in two different conformations, α and β, and if the equilibrium distribution between these depends on *p*H, then the titration curves characteristic of the two conformations are necessarily different. This proposition is readily proved. If (β)/(α) at $p\mathrm{H}_1$ differs from the same ratio at $p\mathrm{H}_2$, then

$$F^0(\beta, p\mathrm{H}_1) - F^0(\alpha, p\mathrm{H}_1) \neq F^0(\beta, p\mathrm{H}_2) - F^0(\alpha, p\mathrm{H}_2)$$

where F^0 is the free energy in a suitable standard state. It follows at once that

$$F^0(\beta, p\mathrm{H}_2) - F^0(\beta, p\mathrm{H}_1) \neq F^0(\alpha, p\mathrm{H}_2) - F^0(\alpha, p\mathrm{H}_1)$$

which is equivalent to saying that the titration curve of β between $p\mathrm{H}_1$ and $p\mathrm{H}_2$ differs from the titration curve of α between the same limits.

The purpose of this paper is to derive the equations which connect these two observable events: the effect of *p*H on conformation and the accompanying changes in titration parameters. The titration curve depends on two properties of the protein molecule's side chains,[1-4] their

* Department of Biochemistry, Duke University, Durham, N. C.

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