proceeds with 80% inversion. Our isolated HOHFU proved to be perfectly stable in the acidic medium and therefore cannot be the source of FU. Consequently it should be the *trans* form with respect to the hydrogens in 5- and 6- position.

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Synthetic Experiments in the Ferrichrome Series*

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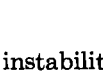
Received June 29, 1964

The amino acid sequences within the cyclic hexapeptide moieties of the ferrichrome compounds have been elucidated. The sequence in ferrichrome (cyclo-triglycyltri-δ-*N*-acethydroxamido-L-ornithinyl) was established through chromatographic examination of partial acid hydrolysates and by the synthetic preparation, from three residues each of glycine and δ-*N*-acetyl-L-ornithine, of a cyclic hexapeptide corresponding to a reduction derivative of the natural product. The sequence in ferrichrome A (cyclo-di-L-serylglycyltri-δ-*N*-acylhydroxamido-L-ornithinyl) was determined through chromatographic analysis of partial acid hydrolysates and through the application of the β-elimination reaction characteristic of serylpeptides.

Previous investigations on the structure of the ferrichrome compounds showed these substances to be ferric trihydroxamates in which the metal-binding center is mounted upon a cyclic hexapeptide platform (Fig. 1) (Emery and Neilands, 1961). The peptide moiety was found to contain three residues of neutral amino acids, which may be glycine alone (ferrichrome) or a combination of 1 mole of glycine with 2 moles of serine (ferrichrome A), together with 3 residues of a new amino acid, δ-*N*-hydroxyornithine (Emery and Neilands, 1961; Rogers and Neilands, 1963). The acyl substituents at the hydroxamate bonds in ferrichrome and ferrichrome A are acetic acid and *trans*-β-methylglutaconic acid, respectively (Emery and Neilands, 1960).

In the formula published previously, the precise sequence of the amino acid residues within the cyclic hexapeptides remained as a major unsolved feature of the proposed structure. In the case of ferrichrome four arrangements could exist, while for ferrichrome A many additional sequence isomers are possible. However, deductions based upon the examination of molecular models coupled with the finding of glycylglycine in partial acid hydrolysates of ferrichrome ruled out the possibility of a completely alternating sequence.

Sequence analysis in the ferrichrome peptides is confronted with several obstacles. In the first place these substances do not seem to be attacked by any of the common proteolytic enzymes. Secondly, the cyclic form is relatively stable compared to the open-chain species, and hence partial acid hydrolysis tends to give either the undegraded peptides or the free amino acids (Bamford and Weymouth, 1955). Finally, the hydroxamic acid groups in the side chains would be expected to be cleaved under conditions which open the cyclic-peptide moiety. This would introduce several additional complications, not the least of which is the



instability of the R—N—H group at neutral *p*H. These considerations prompted a search for a more satisfactory and definitive means of determining the amino acid sequences in ferrichrome and ferrichrome A.

It was reasoned that if the hydroxamic acid linkages could be reduced to the amide level then the resulting stable hexapeptide should, in the case of ferrichrome at least, be available by synthesis. Careful comparison of each of the four synthetic model hexapeptides with fully reduced ferrichrome should then reveal the structure of the natural product. While these experiments were in progress, we were greatly assisted by two developments, namely, (a) a personal communication from O. Mikeš and J. Turková[1] in which they announced the amino acid sequence in the peptide core of the antibiotic albomycin to be cyclo-triseryltri-δ-*N*-acethydroxamidoornithinyl and (b) the appearance of

* This research was supported in part by grants from the Office of Naval Research (222 [39]) and the U.S. Public Health Service (E-4156). It is abstracted from the doctoral dissertation of Samuel Rogers, University of California, Berkeley, 1964.

[1] Institute of Organic Chemistry and Biochemistry, Prague, Czechoslovakia.

a new and effective method for reduction of hydroxamic acids (Gipson *et al.*, 1963). With this information

ferrichrome $\xrightarrow[50\ lb\ H_2]{Raney\ nickel}$ deoxydeferri-ferrichrome $\longleftarrow$ synthesis

in hand we proceeded with the synthesis (Fig. 2) of cyclo-triglycyltri-δ-*N*-acetyl-L-ornithinyl and demonstrated the parallelism of the properties of this substance with those of the reduced derivative of ferrichrome.

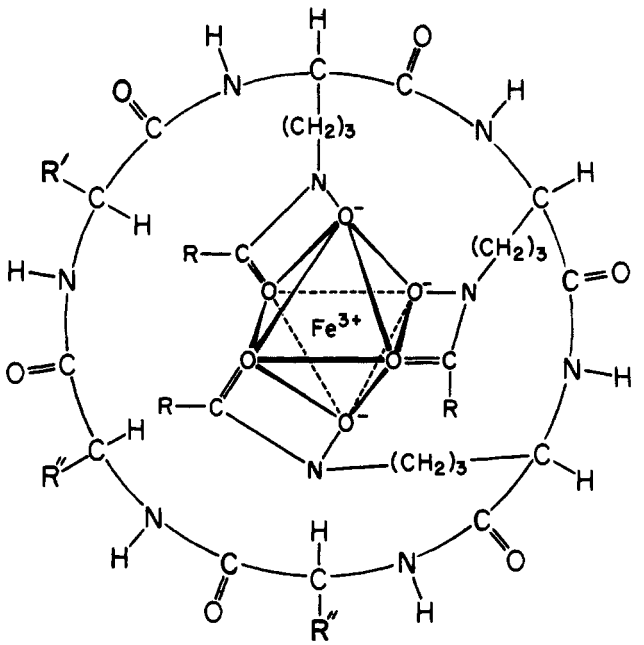
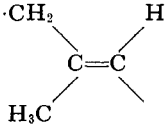


FIG. 1.—*Structures of the ferrichromes* (Emery and Neilands, 1961; Rogers *et al.*, 1963). Ferrichrome: R = CH_3-; R′ = R″ = H-. Ferrichrome A: R = $HOOC{\cdot}CH_2$ H; R′ = H-; R″ = $HOCH_2$-.



The foregoing approach was not feasible for determining the amino acid sequence in ferrichrome A. Here the difficulties inherent in working with serylpeptides precluded synthesis of model hexapeptides and, furthermore, the Raney nickel reduction of ferrichrome A was found to proceed much less smoothly than in the case of ferrichrome. A partial acid hydrolysate gave a very low yield of a peptide which, after purification by successive paper chromatography and electrophoresis, behaved as serylserine. A tripeptide isolated from the supernatant medium of an organism growing on ferrichrome A as a source of carbon and nitrogen was found to have the sequence serylglycyl-δ-*N*-acylhydroxamidoornithine (Rogers *et al.*, 1963). This information, if reliable, would suffice to establish the sequence as cyclo-diserylglycyltri-δ-*N*-acylhydroxamidoornithinyl. For confirmation of this structure, resort was made to the β-elimination reaction for cleavage of serine-containing peptides (Riley *et al.*, 1957; Patchornik and Sokolovsky, 1964). If the amino acid sequence shown in Figure 1 is correct, application of this reaction to ferrichrome A should split out pyruvamide and afford a pyruvoyl N-terminal glycine residue. This postulate was confirmed by use of diphenylphosphorylchloride as leaving group (Y in following reaction).

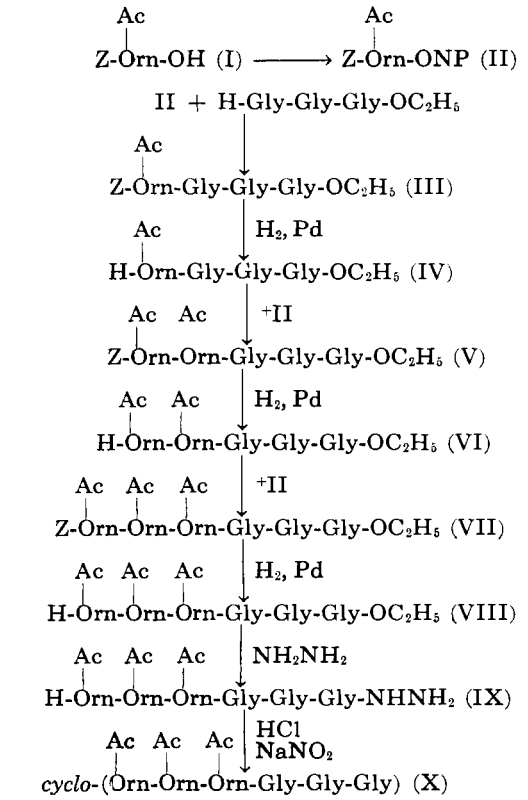
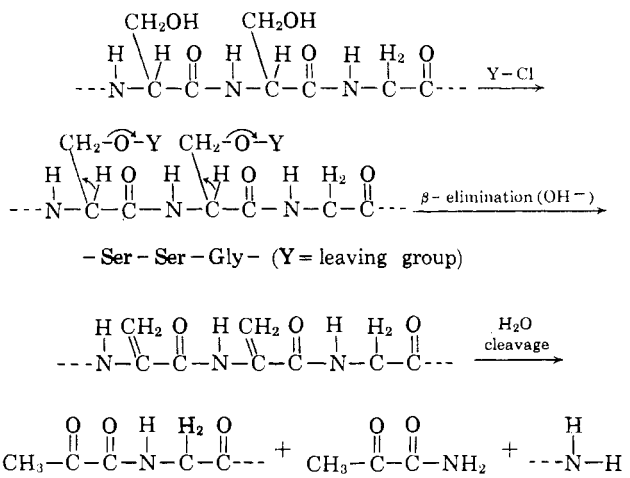


FIG. 2.—Reaction scheme for synthesis of deoxydeferriferrichrome.



In practice it was found expedient to convert the pyruvamide to its 2,4-dinitrophenylhydrazone and the pyruvoylglycylpeptide to its phenylhydrazone; the former was identified directly by thin-layer chromatography (Fig. 3), while the latter was detected as alanylglycine after reduction and partial acid hydrolysis.

It is interesting that the cyclic hexapeptide portions of albomycin, ferrichrome, and ferrichrome A all contain a tripeptide of δ-*N*-hydroxy-L-ornithine (Turková *et al.*, 1963; Rogers *et al.*, 1963). Since the peptide moieties of ferrirhodin, ferrirubin, and ferrichrysin are identical with that of ferrichrome A (Keller-Schierlein, 1963), the amino acid sequence in the former three substances must be as shown in Figure 1. A preliminary account of certain aspects of this work has already been reported (Rogers *et al.*, 1963). In a parallel investigation, R. A. J. Warren[2] in this laboratory has

[2] Doctoral dissertation, University of California, Berkeley.

investigated the mechanism of microbial degradation of the ferrichrome compounds (Warren and Neilands, 1964).

Experimental Procedures and Results

Materials and Methods

All reagents used were of the highest quality obtainable. Recrystallized preparations of ferrichrome and ferrichrome A were secured from *Ustilago sphaerogena* cultures (Neilands, 1952; Garbaldi and Neilands, 1955). Peptides were synthesized by established procedures and elementary analyses were performed by the chemistry department of the University of California at Berkeley. The palladium catalyst was prepared by the method of Greenstein and Winitz (1961). Melting points were measured in capillaries and are uncorrected. The apparent dissociation constants (pK_a values) and neutral equivalents were determined with the Difunctional Recording Titrator, International Instrument Co., Canyon, Calif. (Neilands and Cannon, 1955), in water at 25°. Isolated products were dried *in vacuo* over $CaCl_2$ prior to analysis and characterization.

Partial Acid Hydrolysis of Ferrichrome and Deferriferrichrome

Ferrichrome.—A 14.8-mg (20 μmoles) sample of recrystallized ferrichrome was dissolved in 1 ml 12 N HCl and incubated at 37° for 12 hours. The excess acid was cautiously removed by evaporation under reduced pressure and the residue was dissolved in a drop of water. Chromatography on Whatman No. 1 paper with 80% 1-propanol–20% water as solvent revealed the presence of a component with an R_F (0.10) much less than that of glycine (0.20) and indistinguishable from that of glycylglycine. Like the latter dipeptide, the color after spraying with ninhydrin underwent the yellow → blue transition characteristic of glycine N-terminal peptides.

Deferri-ferrichrome.—The iron was removed from a 22.2-mg (30 μmoles) sample of ferrichrome (Emery and Neilands, 1960), the product was dissolved in 1 ml of concd HCl and incubated at room temperature for 20 hours. The excess acid was cautiously removed by evaporation under reduced pressure and the residue was dissolved in a drop of water. Analysis of the hydrolysate by electrophoresis on Whatman No. 1 paper with pyridine-acetate buffer, *p*H 5.0, for 1 hour at 1000 v showed the presence of ninhydrin-positive components with cathodic migration rates corresponding to those of authentic glycine and glycylglycine. In *p*H 3.2 pyridine-acetate buffer glycylglycine was found to move substantially faster than glycine, thus making possible a more effective separation. The analysis of the unknown was hence repeated at *p*H 3.2, the zone opposite authentic glycylglycine was cut out, eluted, hydrolyzed overnight in 6 N HCl, and shown to contain glycine as the sole ninhydrin-positive component.

Paper chromatographic analysis of the partial hydrolysate in water-saturated phenol containing 1 ml of concentrated ammonium hydroxide per 200 ml of solvent revealed the presence of ninhydrin-positive components with the same R_F as glycine and glycylglycine, namely, 0.31 and 0.46, respectively.

Preparation of Deoxydeferri-ferrichrome (X)

Synthetic.—α-*N*-Carbobenzoxy-δ-*N*-acetyl-L-ornithine (I).—Exactly 1.74 g (0.01 mole) of δ-*N*-acetyl-L-ornithine (Greenstein and Winitz, 1961) was added to 2.1 g (0.025 mole) of $NaHCO_3$ in 20 ml of water and to the vigorously stirred solution was added carbobenzoxy-chloride (1.5 ml) in four portions over a period of 0.5 hour. The reaction mixture was acidified to *p*H 1.7 by careful addition of 5 N HCl. The acid form of the compound was extracted with ethyl acetate and dried over anhydrous Na_2SO_4. The product could not be crystallized in the free acid form and was used directly in the succeeding synthesis. The yield of colorless oil was 2.37 g (8.5 mmoles, 85%).

α-*N*-Carbobenzoxy-δ-*N*-acetyl-L-ornithine *p*-nitrophenyl ester (II).—A 2.37-g. (8.5 mmoles) sample of compound I (acid form) was dissolved in 20 ml of ethyl acetate and the solution was cooled in an ice bath. Exactly 1.52 g (9.5 mmoles) of *p*-nitrophenol and 1.78 g (8.5 mmoles) of dicyclohexylcarbodiimide were then added. The reaction mixture was stirred for 0.5 hour in the ice bath and 1 hour at room temperature, and then were stored overnight at 5°. The precipitate was filtered and washed with a small amount of ethyl acetate (10–20 ml) in order to remove most of the remaining *p*-nitrophenol. The now colorless filter cake was triturated in hot chloroform and filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in a minimum amount of hot ethanol from which the compound crystallized upon cooling. The yield was 2.2 g (60%) of colorless needles with mp 157–158°. Optical rotation $[\alpha]_D^{25} = -36.6°$ (*c* 0.5, DMF).[3]

Anal. Calcd for $C_{21}H_{25}N_3O_7$: C, 58.73; H, 5.40; N, 9.79. Found: C, 58.89; H, 5.31; N, 9.75.

α-*N*-Carbobenzoxy-δ-*N*-acetyl-L-ornithinylglycylglycylglycine ethyl ester (III).—Compound II (2.15 g, 5.0 mmoles) and glycylglycylglycine ethyl ester (1.09 g, 5.0 mmoles) (Rydon and Smith, 1955), in the free-base form, were dissolved in 15 ml of dimethylformamide and held at 37° for 48 hours. Some precipitation of the tetrapeptide usually occurred during the reaction period. Approximately 150 ml of ethyl acetate was then added to the reaction mixture and the peptide precipitated by cooling for several hours at 0°. The product was filtered off, washed with an additional 100 ml of ethyl acetate, and recrystallized from methanol-ether. The yield was 1.95 g (82%) of needles with mp 164–165°. Optical rotation $[\alpha]_D^{25} = -7.1°$ (*c* 1.8, methanol).

Anal. Calcd for $C_{23}H_{33}N_5O_8$: C, 54.42; H, 6.55; N, 13.80. Found: C, 54.49, H, 6.71, N, 13.94.

δ-*N*-Acetyl-L-ornithinylglycylglycylglycine ethyl ester (IV).—Compound III (2.54 g, 5.0 mmoles) was dissolved in 50 ml of methanol and hydrogenated in the presence of palladium catalyst prepared from 0.5 g of palladium chloride. After carbon dioxide evolution had ceased, the catalyst was removed by filtration and the peptide was precipitated in the free-base form by addition of ether. The yield was 1.72 g (92%) of needles with mp 187–188°. The neutral equivalent was 371 (theory, 373). Optical rotation $[\alpha]_D^{25} = +14.9°$ (*c* 1.0, H_2O).

Anal. Calcd for $C_{15}H_{27}N_5O_6$: C, 48.25, H, 7.29; N, 18.76. Found: C, 47.97, H, 7.15; N, 18.59.

α-*N*-Carbobenzoxy-δ-*N*-acetyl-L-ornithinyl-δ-*N*-acetyl-L-ornithinylglycylglycylglycine ethyl ester (V).—Compound II (2.15 g, 5.0 moles) and compound IV (1.87 g, 5.0 mmoles) were dissolved in 25 ml of DMF and the solution was stored at 37° for 48 hours. The pentapeptide, which precipitated during the course of the reaction, was redissolved from time to time by application of heat. Approximately 200 ml of ethyl

Ac
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[3] Abbreviations used in this work: Orn, δ-N-acetyl-L-ornithine; Z, carbobenzoxy group; ONP, *p*-nitrophenyl ester; DMF, dimethylformamide.

acetate was then added to the reaction mixture in order to completely precipitate the pentapeptide. Recrystallization from methanol gave 2.65 g (80%) of prisms with mp 217–218°. Optical rotation $[\alpha]_D^{25} = -7.9°$ (*c* 1.3, DMF).

Anal. Calcd for $C_{30}H_{45}N_7O_{10}$: C, 54.29; H, 6.83; N, 14.77. Found: C, 53.98; H, 6.76; N, 14.66.

δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYLGLYCYLGLYCYLGLYCINE ETHYL ESTER (VI).—Compound V (3.32 g, 5.0 mmoles) was dissolved in 50 ml of methanol and hydrogenated in the presence of palladium catalyst prepared from 1 g of palladium chloride. The catalyst was filtered off after completion of the reduction and the peptide was precipitated out in the free-base form with ether. The pentapeptide free base was a solid which upon drying slowly turned into a glass and was used in this form for subsequent reactions. The compound was determined to be a single component by paper chromatography in several solvents, and by paper electrophoresis. The yield was 2.60 g (96%) of material with neutral equivalent of 547 (theory, 539).

α-*N*-CARBOBENZOXY-δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYLGLYCYLGLYCYLGLYCINE ETHYL ESTER (VII).—Compound II (2.15 g, 5.0 mmoles) and compound VI (2.70 g, 5.0 mmoles) were dissolved in 30 ml of DMF and the solution was stored at 37° for 72 hours. The hexapeptide, which had precipitated during the reaction, was redissolved from time to time by heating. Approximately 200 ml of ethyl acetate was added at the end of the reaction in order to completely precipitate the hexapeptide. Recrystallization from methanol gave 3.4 g (85%) of prisms with mp 192–193°. Optical rotation $[\alpha]_D^{25} = -6.6°$ (*c* 1.0, DMF).

Anal. Calcd for $C_{37}H_{57}N_9O_{12}$: C, 54.20; H, 7.01; N, 15.37. Found: C, 53.98; H, 7.11; N, 15.36.

δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYLGLYCYLGLYCYLGLYCINE ETHYL ESTER (VIII).—Compound VII (2.05 g, 2.5 mmoles) was dissolved in 50 ml of methanol and hydrogenated in the presence of palladium catalyst prepared from 0.5 g of palladium chloride. The catalyst was filtered off after completion of the reduction and the peptide was precipitated in the free-base form by addition of ether. The yield was 1.55 g (92%) of prisms with mp 150°. The neutral equivalent was 696 (theory, 686). Optical rotation $[\alpha]_D^{25} = -17.5°$ (*c* 1.5, H_2O).

Anal. Calcd for $C_{29}H_{51}N_9O_{10}$: C, 50.79; H, 7.50; N, 18.38. Found: C, 50.58; H, 7.44; N, 18.45.

δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYLGLYCYLGLYCYLGLYCINE HYDRAZIDE (IX).—Compound VIII (686 mg, 1 mmole) was dissolved with heating in 25 ml of absolute ethanol, 0.2 ml of hydrazine (95%) was added and the solution was placed in the 37° room overnight. The crystalline hydrazide was broken up, filtered, and washed with 20 ml of absolute ethanol. The yield was 620 mg (92%) of prisms with mp 169–170°. Optical rotation $[\alpha]_D^{25} = -21.2°$ (*c* 1.3, H_2O).

Anal. Calcd for $C_{27}H_{49}N_{11}O_9$: C, 48.27; H, 7.35; N, 22.94. Found: C, 47.81; H, 7.14; N, 22.56.

CYCLO-δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYL-δ-*N*-ACETYL-L-ORNITHINYLGLYCYLGLYCYLGLYCYL (DEOXYDEFERRI-FERRICHROME) (X).—(1) Via dicyclohexylcarbodiimide.—One mmole (686 mg) of the free base of compound VIII was added to 2 ml of 1 N NaOH. After standing at room temperature for 0.5 hour the reaction mixture was acidified to *p*H 5.5 with 1 N HCl. The neutralized material was added to 500 ml of methanol and 1.25 g of dicyclohexylcarbodiimide was added. The reaction mixture was stirred at 4° for 4 days and at room temperature for 3 additional days. The reaction mixture was then evaporated to dryness, the residue dissolved in 25 ml of methanol and 2.5 ml acetic acid was added. The dicyclohexylurea was filtered off and the remaining material was purified by chromatography on Whatman 3 MM paper using the solvent system *t*-butyl alcohol–methyl ethyl ketone–water–triethylamine (40:40:20:4). A band corresponding in R_F to that of reduced natural ferrichrome was detected by application of the chlorine-starch-iodide reagents (V. Kostka, personal communication). The band was eluted; the eluent was evaporated to dryness, dissolved in a minimal amount of methanol and precipitated with acetone. The amount of material obtained in this way was only sufficient for chromatographic analysis.

(2) Via *p*-nitrophenyl ester.—An attempt was made to cyclize the hydrobromide of the *p*-nitrophenyl ester of the linear hexapeptide by use of hot pyridine (95°). Paper chromatographic analysis of the reaction mixture indicated a very low yield of product. However, an intermediate used in the reaction, the *p*-nitrophenyl ester corresponding to compound VII, proved to be very insoluble and was obtained in very crude form. The poor quality of this material may have been responsible for the low yield of the final product.

(3) Via the azide.—All reactions were carried out in the cold room at 0°. One mmole (672 mg) of compound IX was dissolved in 3.1 ml of 1 N HCl, 1.10 mmoles (76 mg) of $NaNO_2$ in 2 ml of H_2O was added, and the solution was gently swirled for 15 minutes. The reaction mixture was poured into 800 ml of water to which 3.7 g of $NaHCO_3$ was added. After stirring for 48 hours at 0°, the *p*H of the solution was adjusted to 5.0 with 2 N HCl. The solution was then evaporated to dryness and the residue extracted three times with 5-ml portions of methanol. The pooled methanol extracts were evaporated; the residue was dissolved in 5 ml of water, passed successively through 1.5 × 6-cm columns of Dowex-1 (acetate) and Dowex-50(H), purified by chromatography as in synthesis (1). The yield averaged 0.06 g (about 10%).

Natural.—A 150 mg (0.2 mmole) sample of ferrichrome was shaken for 6 hours in about 200 ml of methanol with about 1.5 cc of freshly prepared Raney nickel catalyst in a hydrogen atmosphere at 50 psi. The colorless supernatant was decanted through a filter and the sludge was centrifuged and washed with methanol. The combined washings and filtrate were evaporated to a small volume and the product was precipitated by addition of ether. The yield was 69 mg (53%).

PROPERTIES.—Natural and synthetic specimens of compound X were colorless prisms, mp 262–265° (decomp), which were soluble in water or methanol and insoluble in acetone. The R_F values in 1-butanol–acetic acid–water (63:10:27), methyl ethyl ketone–*t*-butyl alcohol–water–diethylamine (40:40:20:4), and 1-butanol-pyridine-water (30:30:30) were 0.43, 0.31, and 0.64, respectively. Partial hydrolysis of natural and synthetic samples of compound X in 12 N HCl at 30° for 1.5 days, with subsequent analysis by paper electrophoresis in 4% formic acid, gave an identical array ("fingerprint") of ninhydrin-positive spots, none of which was ornithine.

The natural and the synthetic compounds gave $[\alpha]_D^{25} = -47°$ (*c* 1.0, H_2O) and $[\alpha]_D^{25} = -48°$ (*c* 1.0, H_2O), respectively. The infrared spectra were essentially identical. The "cross test" for antagonism against albomycin (Řičicová, 1963) was negative for both *Escherichia coli* and *Bacillus subtilis*.

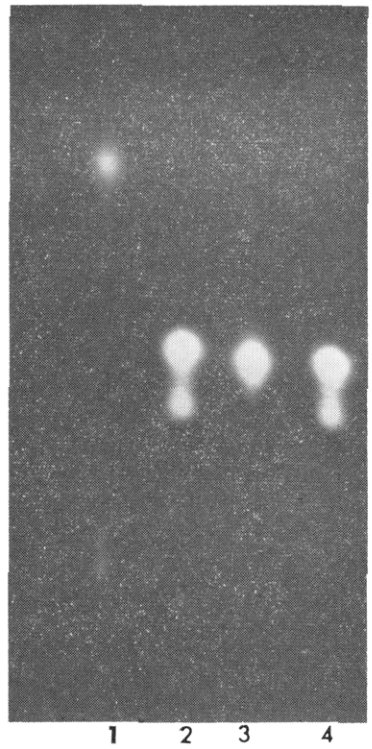

FIG. 3.—Detection of 2,4-dinitrophenylhydrazone of pyruvamide by thin-layer chromatography on silica gel with benzene–acetic acid (85:15) as solvent. The plate was photographed with ultraviolet light and suitably sensitive film. (1) 2,4-Dinitrophenylhydrazine; (2) and (4) mixture of geometrical isomers of 2,4-dinitrophenylhydrazone of authentic pyruvamide; (3) 2,4-dinitrophenylhydrazone from deferri-ferrichrome A.

Anal. Calcd for $C_{27}H_{45}N_9O_9$: C, 50.69; H, 7.09; N, 19.70. Found: Natural—C, 49.6; H, 7.0; N, 19.1; Synthetic—C, 49.7; H, 7.0; N, 19.9.

Partial Acid Hydrolysis of Deferri-ferrichrome A

A 10 mg (0.01 mmole) sample of deferri-ferrichrome A (Emery and Neilands, 1960) was incubated with 1 ml of 12 N HCl at 30° for 24 hours. The excess acid was removed under reduced pressure and the residue was dissolved in a drop of water. The hydrolysate gave positive ninhydrin, tetrazolium, and iron tests. It was examined by chromatography and electrophoresis on paper, according to the following procedures:

Paper Electrophoresis (1 hour at 1200 v; 35 ma).—The hydrolysate was spotted on Whatman No. 1 paper and analyzed together with authentic serylserine in 4% acetic acid (4% acetic acid separates serine from serylserine and glycine, but does not effect a good separation of the latter; in 4% formic acid the free amino acids move relatively faster and serine travels close behind serylserine). A blue, ninhydrin-positive streak stretched the length of the paper, although a distinctly darker zone occurred opposite the serylserine marker. This zone developed ninhydrin color slowly, as did authentic serylserine.

The analysis was continued by spreading about two-thirds of the entire hydrolysate across a sheet of Whatman 3 MM paper. Spots for authentic serylserine, glycine, and serine were placed along the side of the paper; the latter were subsequently cut off and the zone opposite serylserine was eluted with hot water, concentrated, and analyzed with serylserine, glycine, and serine markers. This treatment appeared to eliminate essentially all the free serine. The main batch of the eluate was run again on Whatman 3 MM paper in 4% formic acid in order to remove free glycine. The eluate from this run was separated on Whatman No. 1 paper in 4% acetic acid and the zone opposite serylserine was cut out, eluted, concentrated, and hydrolyzed for 16 hours in 6 N HCl at 100° and analyzed in 4% formic acid as well as in 4% acetic acid. The latter analyses showed strong spots for serine and very much weaker spots for glycine and other trace components. Judging from the intensity of the major nonserine component (i.e., glycine) there could not have been present more than half as much glycine as serine on a molar basis. In view of the known substantial decomposition of serine upon acid hydrolysis, the presence of a serylserine sequence was thus strongly indicated.

Paper Chromatography.—Two chromatograms were run in butanol–acetic acid–water (4:1:5) with descending strips. The solvent was allowed to drip off the paper. Glycine, serine, the original entire hydrolysate, serylserine, and δ-*N*-hydroxyornithine were used as markers. Glycine and serine did not separate under these conditions and moved just ahead of serylserine. The hydrolysate showed a heavy spot opposite serine and glycine, and a definite although weaker spot opposite serylserine.

β-Elimination Reaction Applied to Deferri-ferrichrome A.

Pyruvamide.—The iron was removed from 100 mg (0.1 mmole) of ferrichrome A by the method of Emery and Neilands (1960). The solution of deferri-ferrichrome A was then dried by repeated evaporation from methanol with the aid of the flash evaporator. The dried deferri-ferrichrome A was dissolved in 3 ml of dry pyridine, 270 mg of diphenylphosphorylchloride was added, and the reaction was maintained at 4° overnight. The phosphorylated deferri-ferrichrome A was precipitated with ether and the granular residue was triturated repeatedly and washed with ether. The precipitated material was then dissolved in 1 ml of 1 N NaOH, 5 ml of water was added, and the solution was allowed to stand at room temperature for 0.5 hour. The *p*H was then adjusted to *p*H 7 by addition of 2 N HCl and 0.05 M phosphate buffer and the solution was diluted to a total volume of 20 ml and kept at 100° for 12 hours. The reaction mixture was cooled, evaporated to dryness, and extracted with 5 ml of water. The aqueous extract was cooled, adjusted to *p*H 3.5, and 5 ml of methanol and 75 mg of 2,4-dinitrophenylhydrazine were added. After incubation overnight at 4°, the solution was alternately passed through a short column of Dowex-50 (H) and Dowex-1 (acetate). The yellow effluent was evaporated to a small volume and applied to a thin-layer chromatography plate prepared from silica gel. Development of the plate with benzene–acetic acid (85:15) revealed the presence of a component with the same R_F (0.31) as an authentic (Anker, 1948, mp 214–215°) specimen. The zone corresponding to the main or faster-moving geometrical isomer was eluted from the silica gel with methanol, concentrated, and placed on a fresh thin-layer plate. The analytical results are shown in Figure 3.

Alanylglycine.—A reaction mixture which had undergone the foregoing treatment through the stage of heating at 100° for 12 hours at *p*H 7 was cooled, the *p*H was adjusted to 3.5 with 2 N HCl, and the solution was evaporated to dryness. The residue was taken up in 10 ml of 50% aqueous methanol, 50 mg of phenylhydrazine HCl was added, and the solution was stored at 4° overnight. The entire reaction mixture was reduced with 50 mg of PtO_2 and hydrogen gas (6-hour reaction time). The solution containing the reduction products was evaporated to dryness, dissolved in 2 ml of 12 N HCl, and stored at 37° for 14 hours. The HCl

was removed by repeated evaporation from water under reduced pressure. The product thus obtained was subjected to preparative electrophoresis in *p*H 5.5 pyridine-acetate buffer at 800 v for 7 hours.

The band exhibiting essentially no movement at this *p*H was further purified by electrophoresis in *p*H 3.6 acetic acid–sodium acetate buffer. In this manner it was possible to separate out a single ninhydrin-positive component which had the chromatographic properties of alanylglycine. The eluted substance was hydrolyzed in 6 N HCl and was found to contain only alanine and glycine. After one Edman degradation (Konigsberg and Hill, 1962), glycine was the only remaining ninhydrin-positive material.

ADDED IN PROOF

The amino acid sequence in ferrichrome A has been confirmed by crystallography (Zalkin, A., Forrester, J. D. and Templeton, D. H. [1964], *Science 146*, 261).

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Synthesis of 3,7-Dideoxy-D-*threo*-hepto-2,6-diulosonic Acid: A Study in 5-Dehydroquinic Acid Formation*

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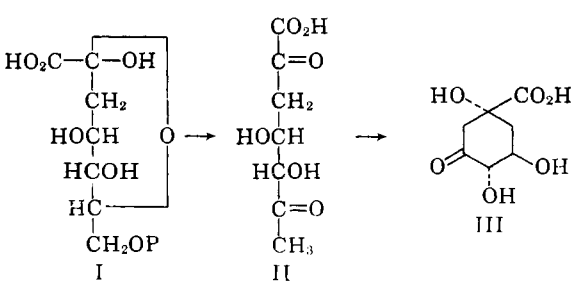
Received July 2, 1964

3,7-Dideoxy-D-*threo*-hepto-2,6-diulosonic acid (II), a postulated intermediate in the enzymic formation of 5-dehydroquinate, was synthesized from glucose via the intermediates V to XII. The key intermediate IX could also be obtained directly from compound VI or from VII. Although compound II was not converted to 5-dehydroquinate enzymically, it was cyclized to this compound by intramolecular aldol condensation at *p*H 11 or in imidazole buffer at *p*H 7, and pure 5-dehydroquinate was isolated. The "physiological conditions" under which compound II is cyclized spontaneously suggest that an enzyme-bound complex of compound II (perhaps in the form of the 6,7-enol) is active in the enzymic formation of 5-dehydroquinate from 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (I).

Earlier investigations in this laboratory have shown that the cyclization reaction in the biosynthesis of the aromatic amino acids in *Escherichia coli* is the conversion of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (I) to 5-dehydroquinate (III) (Srinivasan *et al.*, 1963; Sprinson, 1960). The enzymic activity responsible for this conversion could not be fractionated into independent reactions, and evidence was presented to support the view that there were no free intermediates between substrate and product. These results were rationalized by a reaction scheme in which oxidation of compound I at C-5 facilitated elimination of orthophosphate, and reduction at C-5 then yielded 3,7-dideoxy-D-*threo*-hepto-2,6-diulosonic acid (II). The oxidation-reduction was assumed to be mediated by NAD, which was required for activity. An internal aldol condensation of compound II was postulated to yield III. Although the intermediates appeared to be enzyme-bound, it seemed worthwhile to synthesize compound II in order to test its chemical properties and its reactivity in the presence of 5-dehydroquinate synthetase.

The plan for the synthesis of compound II (see struc-



* This work was supported by grants from the American Cancer Society, the American Heart Association, the National Institutes of Health of the U. S. Public Health Service, and the National Science Foundation.

† Career Investigator of the American Heart Association.