Structures of the Naturally Occurring Hydroxamic Acids, Fusarinines A and B*

Jane M. Sayer and Thomas F. Emery

ABSTRACT: Two hydroxamic acids produced by *Fusarium roseum* have been isolated and their structures were determined. Total acid hydrolysis of these substances yielded only δ-*N*-hydroxyornithine and *cis*-3-methylpent-2-eno-5-lactone, which were shown to be in a 1:1 ratio in the original compounds. Mild hydrolysis yielded fusarinine (Emery, T. (1965), *Biochemistry 4*, 1410). Periodate cleavage of the hydroxamate bonds of fusarinine A, followed by catalytic hydrogenation, gave a product chromatographically identical with chemically synthesized 5-*O*-(L-ornithyl)-*dl*-3-methylpentanoic acid. These findings, supported by titrimetric evidence, indicated that fusarinines A and B are composed of fusarinine units joined by very labile α-amino ester bonds between the carboxyl group of δ-*N*-hydroxyornithine and the hydroxyl of the *cis*-5-hydroxy-3-methylpent-2-enoic acid residue of the adjacent fusarinine unit. The ease of lactonization of 5-hydroxy-3-methylpentenoic acid enabled us to remove this terminal acyl group. Acetylation of the hydroxylamino group thus exposed, using [^{14}C]acetic anhydride, and determination of the ^{14}C to ornithine ratio in the product provided a method of molecular weight determination. Fusarinine A was shown by this method to contain two fusarinine units and fusarinine B, three fusarinine units. The stability of the iron chelate of fusarinine B supported this conclusion. A third hydroxamate, fusarinine C, is believed to be a cyclic form of fusarinine B. All of these substances showed slight growth-factor activity with *Arthrobacter* JG-9.

These compounds represent a new class of naturally occurring hydroxamic acids in which the hydroxamate subunits are joined by ester linkage rather than the usual peptide bonds.

The naturally occurring hydroxamic acid, fusarinine, was previously shown to be δ-*N*-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-δ-*N*-hydroxyornithine (Emery, 1965). In all other hydroxamic acids containing δ-*N*-hydroxyornithine, three residues of this amino acid occur in peptide linkage, and the three side-chain hydroxylamino groups are acylated with acetate or a mevalonic acid derivative to form a trihydroxamic acid (Neilands, 1966). Fusarinine itself is found in such peptide linkage in ferrirhodin.

Several hydroxamic acids in addition to fusarinine are found in the culture fluid of *Fusaria*. The purpose of this paper is to show that these compounds are dimers and trimers of fusarinine linked by ester rather than peptide groups. This structure is unique among the natural hydroxamic acids and is also an example of the relatively rare class of amino acid esters that are known to occur in nature.

Results

The fusarinine compounds are amorphous, hygroscopic, and yellow or light brown. Although other natural hydroxamates have been isolated in yields approaching 0.5 g/l. of culture fluid (Neilands, 1966), only 25–30 mg of fusarinine A could be obtained in a pure state from 1 l. of *Fusarium* culture fluid, and yields of fusarinine B were even lower. These low recoveries were undoubtedly due in part to the instability and difficulty of purification of these substances. The compounds were obtained as the acetate salts by lyophilization from the acetate-containing buffer used in their electrophoretic purification. After final purification they gave single ninhydrin- or ferric chloride positive spots upon electrophoresis. Paper chromatography of fusarinines A and B in several solvent systems also gave single spots. Solvent system d gave the most satisfactory resolution of fusarinine, fusarinine A, and fusarinine B.

Total Hydrolysis. Hydrolysis of fusarinine A with 6 N hydrochloric acid gave a ninhydrin- and tetrazolium-positive compound with electrophoretic mobility identical with that of δ-*N*-hydroxyornithine obtained from fusarinine. The presence of δ-*N*-hydroxyornithine in fusarinine A was confirmed by the identification of ornithine as the only amino acid produced upon reductive hydrolysis of the hydroxamate with hydriodic acid (Emery and Neilands, 1961). Ornithine was identified by ascending paper chromatography in solvent e (first dimension) and water-saturated collidine (second

* From the Department of Biochemistry, Yale University, New Haven, Connecticut. *Received September 25, 1967.* This publication is abstracted from a dissertation presented by J. M. S. in partial fulfillment of the requirements for the Ph.D. degree. Financial support from the National Institutes of Health, U. S. Public Health Service (GM-09709-05), is gratefully acknowledged. During this investigation J. M. S. was a trainee of the U. S. Public Health Service (1964–1965) and a predoctoral fellow of the National Institute of General Medical Sciences (1965–1967).

dimension). The L configuration of this amino acid in fusarinine A was shown by the close correspondence of the optical rotatory dispersion spectrum between 250 and 350 mμ of ornithine from fusarinine A with that of authentic L-ornithine. Ornithine was also shown chromatographically to be the sole amino acid obtained upon hydriodic acid hydrolysis of fusarinine B.

Identification of Δ^2*-HMP-lactone*[1] *from Fusarinine A.* The presence of an α,β-unsaturated acyl group in fusarinines A–C was suggested by their ultraviolet spectra, which closely resembled that of fusarinine. Each of the three new compounds exhibited only one peak in the ultraviolet region with a λ_{max} between 210 and 220 mμ. The acyl portion of fusarinine A was isolated after cleavage of the hydroxamate bonds by either periodic acid oxidation or hydrolysis with dilute hydrochloric acid (Emery, 1965). In order to ensure the complete degradation of the molecule, fusarinine A was hydrolyzed in water at 100° before periodate or hydrochloric acid treatment. Fusarinine A subjected to either of these procedures yielded a single ether-soluble product, which was identical in gas chromatographic behavior and infrared spectrum to synthetic Δ^2-HMP-lactone (Emery, 1965; Cornforth *et al.*, 1958).

The concentration of Δ^2-HMPA in solutions of fusarinine A and fusarinine (control) was determined by ultraviolet spectroscopy. The molar extinction coefficient of the methyl ester of Δ^2-HMPA (1.2×10^4) (Cornforth *et al.*, 1958) was assumed to be the same as the extinction coefficient of this acid in hydroxamic acid linkage in the fusarinines at their wavelengths of maximal absorption. The validity of this assumption was shown by the correct ratio of Δ^2-HMPA to ornithine found in the known compound, fusarinine, by this method. Ornithine assays were performed by the method of Chinard (1952) after hydriodic acid hydrolysis of a sample of each solution. Similar procedures were carried out using fusarinines B and C. Table I summarizes the results obtained from these experiments.

Hydrolysis in Water. Fusarinine A is very sensitive to attack by hydroxylic solvents. Heating an aqueous solution of fusarinine A (pH 5.5–6.0) at 100° for 30 min converted it almost completely to a neutral compound which was identified as fusarinine by cochromatography in several solvent systems.

This unusual susceptibility to hydrolysis was not restricted to fusarinine A. Upon heating in aqueous solution for 5 min, fusarinine B yielded two compounds with the electrophoretic and chromatographic behavior of fusarinine and fusarinine A. Only fusarinine was observed on a chromatogram of the compound after 35 min of heating. Upon similar treatment, fusarinine C gave three compounds with electrophoretic mobilities corresponding to fusarinine, fusarinine A, and fusarinine B. We concluded that all three compounds consisted only of fusarinine units joined by an extremely labile bond. An ester bond was suggested by the presence in the infrared spectrum of fusarinine A of a well-defined carbonyl band at 1740 cm^{-1}, which was not observed in the spectrum of fusarinine.

TABLE I: Ratio of Δ^2-HMPA to Ornithine in the Fusarinine Compounds.

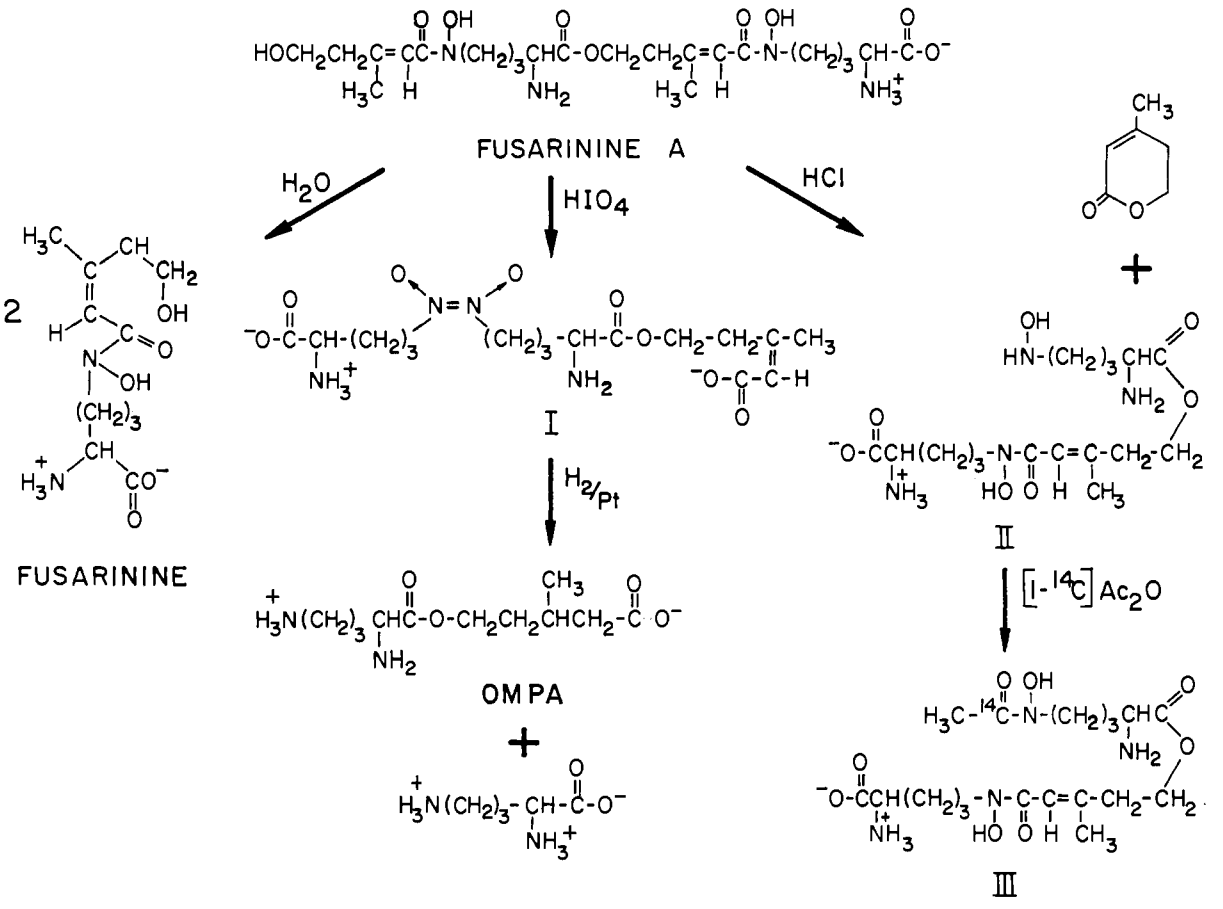
Compound	λ_{max} (mμ)	Δ^2-HMPA[a] (μmoles/ml)	Ornithine (μmoles/ml)	Δ^2-HMPA:Orn Ratio
Fusarinine[b]	220	1.39	1.18	1.2:1.0
Fusarinine A	215	0.92	0.76	1.2:1.0
Fusarinine B	215	0.90	0.78	1.1:1.0
Fusarinine C	210	0.23	0.21	1.1:1.0

[a] Based on a molar extinction coefficient of 1.2×10^4 (see Results). [b] Control; theoretical value of Δ^2-HMPA:Orn is 1.0:1.0.

Identification of OMPA from Fusarinine A. In order to establish definitely the presence of an ester bond in fusarinine A, the isolation of a degradation product containing this group was undertaken. The reaction sequence employed for this purpose is illustrated in Scheme I. Periodate oxidation of the hydroxamic acid bonds was chosen as the method most likely to effect a partial cleavage of the molecule while leaving the sensitive ester bond intact. Compound I produced by periodic acid oxidation is an ester of dimeric 2-amino-5-nitrosopentanoic acid. Catalytic hydrogenation of I should reduce the nitroso group to an amino function and saturate the double bond yielding 5-*O*-(L-ornithyl)-*dl*-3-methylpentanoic acid (OMPA). Paper electrophoresis of the products from fusarinine A followed by chromatography in solvent a in the second dimension indicated the presence of several ninhydrin-positive compounds including proline, ornithine, ornithine lactam, and a cationic compound with electrophoretic mobility slightly less than that of ornithine. This last compound was purified by electrophoresis and compared by chromatography with chemically synthesized OMPA.

Chemically synthesized OMPA showed a carbonyl-absorption band at 1740 cm^{-1}. Titration of the dihydrochloride with potassium hydroxide at 1° gave a pK_1' for the carboxyl group of 4.0; values pf pK_2' (α-amino) and pK_3' (δ-amino), estimated from the titration curve below pH 10 by the method described by Albert and Serjeant (1962), were 7.8 and 10.3 ± 0.1, respectively. The equivalent weight based on the carboxyl group was 320 (theoretical, 319), and the ratio of carboxyl group to ornithine was 1.2:1.0. OMPA was quite unstable in the presence of base. After 2 hr at room temperature in ethanol–acetonitrile solution containing triethylamine, OMPA was completely converted to ornithine lactam.

[1] Abbreviations used: Δ^2-HMP-lactone, *cis*-3-methylpent-2-eno-5-lactone; Δ^2-HMPA, *cis*-5-hydroxy-3-methylpent-2-enoic acid; OMPA, 5-*O*-(L-ornithyl)-*dl*-3-methylpentanoic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.



SCHEME I: Structure of Fusarinine A and Methods of Chemical Degradation.

Synthetic OMPA and the compound isolated after periodate cleavage and catalytic hydrogenation of fusarinine A were subjected to chromatography in four solvent systems (Experimental Section). The observed R_F values are listed in Table II. The identification of a degradation product of fusarinine A as OMPA definitely established the presence of an ester link between the carboxyl of a δ-*N*-hydroxyornithine residue and the hydroxyl of a Δ^2-HMPA residue in fusarinine A.

TABLE II: Chromatographic Comparison of Ester from Fusarinine A and Chemically Synthesized OMPA.

Solvent System	R_F Values	
	Synthetic OMPA	Ester from Fusarinine A
a	0.19[a]	0.20
b	0.69	0.70
c	0.92	0.91
d	0.18[a]	0.17

[a] R_F of major spot.

Replacement of Terminal Δ^2-HMPA with [1-^{14}C]-Acetate; the Size of the Fusarinine A and B Molecules. An attempt was made to determine the number of fusarinine units in fusarinine A by molecular weight determination. However, the lability of the ester bond made this approach unsuccessful. Vapor pressure osmometry in methanol at 37° gave erroneously low values of the molecular weight as a result of methanolysis of the compound. The methyl ester of fusarinine was isolated and identified by electrophoretic comparison with the ester produced by the reaction of fusarinine with diazomethane. This susceptibility to methanolysis was also indicated by the isolation of fusarinine methyl ester as a contaminant of fusarinine B when methanol was used to extract the fusarinines from the dried *Fusarium* culture medium. When Methyl Cellosolve was used in place of methanol as the extraction solvent, this impurity was not found.

The problem of the molecular size of fusarinine A was solved by a chemical method based on the selective replacement of the terminal Δ^2-HMPA residue with a labeled group making possible an end group analysis. The reactions involved in this procedure are illustrated in Scheme I for the case of fusarinine A. Because of its ease of lactonization, the "hydroxyl-terminal" Δ^2-HMPA residue of fusarinine A or B was easily removed

by mild acid hydrolysis under conditions which did not cleave other bonds in the molecule. Heating fusarinine A with 0.5 N hydrochloric acid resulted in complete conversion to a product (II) with a free hydroxylamino group, as demonstrated by increased electrophoretic mobility and positive tetrazolium reaction. The free hydroxylamino group was selectively acetylated with radioactive acetic anhydride, yielding a derivative (III) in which the terminal Δ^2-HMPA group of the parent compound had been replaced by [1-^{14}C]acetate. Reappearance of a ferric chloride positive, tetrazolium-negative spot with a mobility characteristic of the parent compound demonstrated completion of the acetylation reaction. Figure 1 shows typical electrophoretic patterns obtained after mild hydrochloric acid hydrolysis and acetylation of fusarinine A. The radioactive material (III), which corresponded in mobility to fusarinine A, was eluted from the paper. Since hydroxylamines are known to react with excess acylating agents yielding *N*,*O*-disubstituted products (Prelog and Walser, 1962), it was necessary to treat the eluted material with alkali (Jencks, 1958) to remove *O*-acetyl groups prior to the determination of radioactivity. Fusarinine B underwent reactions analogous to those of fusarinine A when subjected to similar procedures. The results of acetate and ornithine determinations performed on these fusarinine derivatives, in which the terminal acyl group had been replaced by [^{14}C]acetyl, are summarized in Table III. [1-^{14}C]Acetyl-δ-*N*-hydroxyornithine, prepared as a

TABLE III: Ratios of Acetate to Ornithine in Fusarinines after Replacement of Terminal Acyl Group with [1-^{14}C]Acetate.

Parent Compound	Acetate[a] (mμmoles/ml)	Ornithine (mμmoles/ml)	Acetate:Orn Ratio
Fusarinine[b]	1390	1360	1.00:0.98
Fusarinine A	455	940	1.00:2.06
Fusarinine B	46	110	1.00:2.39

[a] Specific activity = 8.8×10^3 dpm/μmole of acetate. [b] Control; theoretical value of acetate:Orn ratio is 1.00:1.00.

control from fusarinine, is also shown. From the observed acetate to ornithine ratios, it was concluded that fusarinine A consists of two ester-linked fusarinine units. Fusarinine B did not yield such clear-cut results; however, since significantly more than 2 moles of ornithine were found per mole of acetate, the conclusion was drawn that this compound was most probably trimeric. This conclusion was also supported by the stability of the ferric chelate of fusarinine B (see below).

Titration of Fusarinine A. Fusarinine A acetate was titrated in 80% (v/v) Methyl Cellosolve at 1°, with 1 N aqueous hydrochloric acid or potassium hydroxide as the titrant. Neither the α-amino nor the hydroxamic acid groups were titrated because of the instability of the ester group above pH 10. The ratio of ornithine to α-carboxyl group in fusarinine A was 2.00:1.07. The following pK_a' values were found: pK_1', 3.6 (α-carboxyl); pK_2', 6.4 (acetic acid); and pK_3', 8.1 (amino group α to ester). The latter two values were calculated from the titration curve by the application of the formulas described by Albert and Serjeant (1962) for the separation of two overlapping ionization constants. A model mixture, consisting of equimolar quantities of leucine, leucine ethyl ester, and acetic acid, gave pK_a' values in 80% Methyl Cellosolve which resembled those observed for fusarinine A acetate. They were as follows: pK_1', 4.0 (α-carboxyl of leucine); pK_2', 6.8 (acetic acid); and pK_3', 8.0 (amino group of leucine ethyl ester). Elemental analysis[2] of a sample of fusarinine A acetate agreed well with the proposed dimeric structure, shown in Scheme I.

Anal. Calcd for $C_{22}H_{38}N_4O_9 \cdot C_2H_4O_2$ (562.6): C, 51.24; H, 7.53; N, 9.96. Found: C, 51.37; H, 7.67; N, 9.72.

Ferric Chelates of Fusarinines A and B. The ferric chelates of fusarinines A and B were prepared by addition of small portions of ferric chloride to a solution of fusarinine A or B until no further increase in optical density at 440 mμ was observed. The absorption maximum of ferric fusarinine B at 440 mμ was found to be quite insensitive to changes in pH, whereas the λ_{max} of ferric fusarinine A was shifted from 430 mμ at pH 8 to 475 mμ at pH 2. Insensitivity of the spectrum to pH changes is a characteristic of known ferric trihydroxamates (Schwarzenbach and Schwarzenbach, 1963). This behavior therefore lends support to the formulation of fusarinine B as a trihydroxamic acid containing three ester-linked fusarinine units per molecule.

Growth Factor Activity toward Arthrobacter JG-9. Fusarinine in concentrations as high as 100 μg/ml was found by Emery (1965) to be inactive in promoting growth of *Arthrobacter* JG-9. Similar tests with fusari-

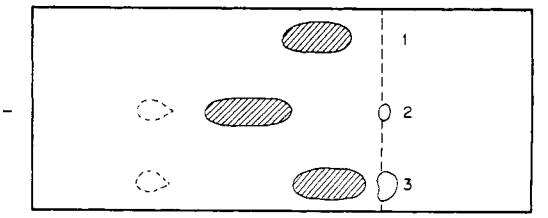


FIGURE 1: Paper electrophoresis of products formed from fusarinine A. Sample 1, fusarinine A; sample 2, fusarinine A after 8 min at 100° in 0.5 N HCl; sample 3, acetylation of 2 with acetic anhydride. Spots were developed by spraying with 5% $FeCl_3$. Broken line indicates the origin.

[2] By Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

nines A–C indicated that all three of these compounds gave a growth response at a concentration of 100 μg/ml, which is approximately 100 times the concentration required to give a similar response with ferrichrome. The low activity could be attributed at least in part to the instability of the compounds under the conditions of the growth-factor tests. Samples of fusarinines A–C, after incubation at 37° for 24 hr in sterile *Arthrobacter* JG-9 medium, showed almost complete degradation to the inactive compound, fusarinine.

Discussion

The novel structures of many of the naturally occurring hydroxamic acids have necessitated the utilization of unusual methods in structural determination. This has been especially true in the present work in which the fusarinine A molecule was attacked at three points: (1) cleavage of the labile α-amino ester bond to yield fusarinine; (2) cleavage of the terminal acyl group as the lactone, and its replacement with radioactive acetate to enable a molecular weight determination; and (3) cleavage of the two hydroxamate bonds with periodate to yield the ornithyl ester moiety upon hydrogenation. These reactions are summarized in Scheme I, and the structure of fusarinine A shown is consistent with these results. Because of the extreme sensitivity of the α-amino ester bond, only the first of the above reactions was quantitative. The finding that no products other than fusarinine were formed on very mild hydrolysis of fusarinine A at a pH near neutrality indicated that the compound must be composed solely of fusarinine units and that no additional substituents could be present on any of the functional groups. This conclusion was supported by the titration data and elemental analysis. Monohydroxamic acids and dihydroxamic acids are quite rare in nature, and it is possible that the presence of fusarinine and fusarinine A in *Fusaria* cultures is a consequence of the spontaneous hydrolysis of the labile ester bonds of the trihydroxamic acid, fusarinine B. Such a breakdown was seen to occur under conditions of the fermentation.

We believe that fusarinine C is a cyclic form of fusarinine B in which the carboxyl-terminal end of fusarinine B is esterified to the hydroxyl-terminal end. Owing to lack of sufficient material for adequate characterization, this conclusion is mainly speculative. It is interesting, however, that an identical substance, named fusigen, has been recently reported to be produced by *Fusarium cubense* (Diekmann and Zähner, 1968). The trihydroxamic acid nature of fusarinine C is supported by its hydrolysis to fusarinine B, whose ferric chelate displays the stability characteristic of trihydroxamic acids.

Most of the known hydroxamic acids may be classified in two groups: the ferrichrome type, which are cyclic hexapeptides containing δ-*N*-hydroxyornithine, and the ferrioxamine type, which contain 1-amino-ω-hydroxylaminoalkanes and in which the hydroxamate groups are in a linear array. The fusarinines contain δ-*N*-hydroxyornithine but, because of the unusual ester linkage, the hydroxamate groups are in linear sequence; these compounds are thus unique among the naturally occurring hydroxamic acids. Another similarity between the ferrichromes and fusarinines is that there are ten atoms between adjacent hydroxamate groups in these compounds while nine, or more rarely eight, atoms are found between hydroxamate groups in the ferrioxamines. In all of these compounds the ferric ion is octahedrally coordinated by the three bidentate hydroxamate groups. The finding of the ornithyl ester link in the fusarinines adds these compounds to the short list of natural products known to contain the α-amino ester function.

Evidence has previously been presented that δ-hydroxamic acid derivatives of ornithine are precursors of trihydroxamic acids of the ferrichrome type (Emery, 1966). Fusarinine may then be a precursor of ferrirhodin, a compound containing three fusarinines in peptide linkage. The mechanism of biosynthesis of microbial peptides is not known, but it is not unlikely that carboxyl-activated intermediates are involved. The carboxyl group of fusarinine engages in peptide formation (ferrirhodin) or ester formation (fusarinines A and B) depending on the organism, and it will be interesting to determine if the same activated intermediate is involved in both cases.

Experimental Section

General Methods. Ascending paper chromatography was performed on Whatman No. 1 paper in the following solvent systems: (a) 1-butanol–ethanol–water (4:1:5) (upper phase), (b) pyridine–water (65:25), (c) methanol–water (4:1), (d) 1-butanol–acetic acid–water (100:12:25), and (e) phenol–water (4:1), containing 0.1 mg/ml of 8-hydroxyquinoline. Spots were routinely developed with 0.5% ninhydrin in acetone or 5% aqueous ferric chloride. Paper electrophoresis was carried out in pyridine–acetic acid–water (14:10:930) (Emery, 1965) at a potential of 28 v/cm. Compounds containing ^{14}C were located on paper electropherograms with a Vanguard 880 automatic scanner.

Visible and ultraviolet spectra were obtained with a Bausch and Lomb 505 recording spectrophotometer. Infrared spectra were obtained with a Beckman IR 5 or a Perkin-Elmer 137 infrared spectrophotometer. Measurements of pH and potentiometric titrations were performed using a Corning Model 12 pH meter equipped with a semimicro combination electrode.

Isolation of Fusarinines. Growth of the organism (*F. roseum*, ATCC 12822) and isolation of the fusarinines followed the procedure of Emery (1965) with the following modifications: the medium initially contained as carbohydrate source 10 g/l. of glucose, and after 2 days of growth an additional 10 g/l. of glucose was added. The fermentation was terminated after 4–5 days, and after concentration of culture fluid either methanol or Methyl Cellosolve was used to extract the fusarinines. The components of the mixture were separated by electrophoresis on Geon 427 (B. F. Goodrich Chemical Co., Cleveland, Ohio). If necessary, further purification was effected by a second electrophoresis on Geon using less material. In order of increasing cationic mobility

the compounds are fusarinine (neutral), fusarinine A, fusarinine B, and fusarinine C. Fusarinine A was chromatographically homogeneous and was used without further purification. Fusarinine B from methanol extracts was separable into two components in solvent system d and was purified by ascending chromatography in this system.

Replacement of Terminal 5-Hydroxy-3-methylpent-2-enoic Acid Residue with [1-^{14}C]Acetate. Fusarinine A (5 mg) or fusarinine B (2 mg) was hydrolyzed at 100° in 50 μl of 0.5 N hydrochloric acid for 8 min. To the acidic solution was added 2–5 μl of [1-^{14}C]acetic anhydride with a specific activity of 8.8×10^3 dpm/μmole of acetate. Acetylation was allowed to proceed at 0° for 1.5 hr. The excess anhydride was then extracted with ether and the reaction mixture was subjected to paper electrophoresis for 1.5 hr. The radioactive band corresponding in mobility to the original compound was eluted with water. Each eluate was lyophilized and the residue was dissolved in 2 ml of water.

To remove *O*-acetyl groups, 0.5 ml of each of the above solutions was treated with 0.25 ml of 3.5 N sodium hydroxide, and the solution was kept at room temperature for 15 min (Jencks, 1958). After acidification with 0.1 ml of 8 N hydrochloric acid, each sample was diluted with water, lyophilized, and heated for 1.5 hr *in vacuo* at 60°. The resulting material was dissolved in water and the volume was adjusted to 1.0 ml. Radioactivity in an 0.8-ml sample was determined using a Beckman CPM-100 liquid scintillation counter. The scintillation medium was 15 ml of modified Bray's solution (dioxane containing 20% naphthalene, 1% PPO, and 0.025% POPOP) to which was added 0.5 g of Cab-O-Sil (Cabot Corp., Boston, Mass.). The efficiency of the counter was 79–80% for ^{14}C with a background of 30 cpm. The standard error was less than 5% for all radioactivity measurements.

5-O-(L-Ornithyl)-dl-3-methylpentanoic Acid (OMPA). FROM FUSARININE A. Fusarinine A (5.9 mg) was treated at 0° with 1 ml of 0.05 M periodic acid for 5 min. Dowex 1 (formate) was then added in small portions until a sample of the supernatant no longer gave a color with potassium iodide and starch. After removal and water washing of the resin, the combined solution and washings were acidified with 0.5 ml of glacial acetic acid and diluted to 5 ml. The solution was hydrogenated at 1 atm over platinum oxide catalyst at 0°. The course of the reaction was followed by observing the optical density of a tenfold dilution of the reaction mixture at 265 mμ. The optical density fell from an initial value of 0.55 to 0 in 4 hr. The catalyst was removed by filtration and the filtrate was treated with 25 μl of 1 N hydrochloric acid and lyophilized. The products were subjected to paper electrophoresis, and the ninhydrin-positive component with a cationic mobility slightly less than that of ornithine was isolated.

CHEMICAL SYNTHESIS. *N,N'*-Dicarbobenzyloxy-L-ornithine was prepared by the method of Synge (1948) from 2.5 g of L-ornithine monohydrochloride and 7.2 ml of carbobenzyloxy chloride. The yield of recrystallized material melting at 110–112° (uncor) was 3.6 g (60%).

dl-3-Methylpentano-5-lactone was prepared by treatment of 22.5 g of β-methylglutaraldehyde with aqueous sodium hydroxide following the procedure of Longley *et al.* (1952). The yield was 11.8 g (52%) of material boiling at 78–81° at 2.5 mm. Sodium *dl*-5-hydroxy-3-methylpentanoate was prepared by saponification of 6.4 g of the lactone with 75 ml of 1 N sodium hydroxide. The basic solution was brought to a pH of approximately 7.8 with 1 N hydrochloric acid and evaporated to dryness. After repeated evaporation with absolute ethanol and drying over P_2O_5 *in vacuo*, a powder was obtained which was dissolved in hot *N,N*-dimethylformamide. Insoluble sodium chloride was removed by centrifugation. Storage of the resultant solution at 7° resulted in precipitation of sodium *dl*-5-hydroxy-3-methylpentanoate as a fine powder, which was collected by centrifugation, washed with ether, and dried *in vacuo* at 100° for 2 hr. The yield was 6.3 g (73%), mp 112–115° (uncor). Potentiometric titration with 1 N hydrochloric acid gave an equivalent weight of 160 (theoretical, 154).

A modification of the carbonyldiimidazole method described by Staab (1959) and Brockmann and Lackner (1960) was used for ester formation. *N,N'*-Dicarbobenzyloxy-L-ornithine (0.82 g) was dissolved in 30 ml of dry, amine-free *N,N*-dimethylformamide and 0.32 g of *N,N'*-carbonyldiimidazole was added. The reaction mixture was stirred for 30 min at room temperature, and a solution of 0.31 g of sodium *dl*-5-hydroxy-3-methylpentanoate in *N,N*-dimethylformamide was added dropwise to the stirred acylimidazole solution. After the addition was complete the mixture was stirred for 1 hr and allowed to stand overnight at room temperature. All the foregoing steps were carried out in an atmosphere of dry nitrogen.

The reaction mixture was evaporated *in vacuo* at 45°, and the residue was treated with 20 ml of cold 1 N hydrochloric acid and extracted three times with ether. Evaporation of the ether yielded 1.0 g of a yellow oil which was dissolved in 25 ml of absolute ethanol.

A 15-ml portion of the ethanolic solution was acidified with 1.5 ml of glacial acetic acid and hydrogenated over palladium–charcoal (10%) for 2 hr at 35 psi. The progress of the hydrogenolysis was followed by electrophoresis of samples of the reaction mixture; the reaction was complete when initially formed, neutral, ninhydrin-positive material (δ-*N*-monocarbobenzyloxyornithyl ester and/or δ-*N*-monocarbobenzyloxyornithine) was no longer found. The catalyst was removed by filtration and 0.2 ml of concentrated hydrochloric acid was added to the filtrate. The solution was concentrated *in vacuo* and applied to Whatman 3MM paper. Ascending chromatography was carried out at room temperature in 1-butanol–ethanol–0.5 N HCl (4:1:5) (upper phase). The faster moving of the two principal ninhydrin-positive components was eluted with dilute hydrochloric acid. The slower moving band, which corresponded to ornithine, was not isolated. The eluate was lyophilized to yield a glass which became gummy upon exposure to air; after drying *in vacuo* over P_2O_5 at room temperature the product was ob-

tained as an extremely hygroscopic yellow powder in a yield of 20–25 mg.

References

Albert, A., and Serjeant, E. P. (1962), Ionization Constants of Acids and Bases, London, Methuen, pp 51–56.
Brockmann, H., and Lackner, H. (1960), *Naturwissenschaften 47*, 230.
Chinard, F. P. (1952), *J. Biol. Chem. 199*, 91.
Cornforth, J. W., Cornforth, R. H., Popják, G., and Gore, I. Y. (1958), *Biochem. J. 69*, 146.
Diekmann, H., and Zähner, H. (1968), *Helv. Chim. Acta* (in press).
Emery, T. (1965), *Biochemistry 4*, 1410.
Emery, T. (1966), *Biochemistry 5*, 3694.
Emery, T., and Neilands, J. B. (1961), *J. Am. Chem. Soc. 83*, 1626.
Jencks, W. P., (1958), *J. Am. Chem. Soc. 80*, 4581.
Longley, R. I., Jr., Emerson, W. S., and Shafer, T. C. (1952), *J. Am. Chem. Soc. 74*, 2012.
Neilands, J. B. (1966), *Struct. Bonding (Berlin) 1*, 59.
Prelog, V., and Walser, A. (1962), *Helv. Chim. Acta 45*, 631.
Schwarzenbach, G., and Schwarzenbach, K. (1963), *Helv. Chim. Acta 46, 1390.*
Staab, H. A. (1959), *Angew. Chem. 71*, 194.
Synge, R. L. M. (1948), *Biochem. J. 42*, 99.

Spontaneous Reactions of 1,3-Substituted 1,4-Dihydropyridines with Acids in Water at Neutrality. II. Nuclear Magnetic Resonance Studies*

Kyoung S. Choi† and Spyridon G. A. Alivisatos

ABSTRACT: Spin–spin coupling assignments for the ring protons in 1-methyl-1,4-dihydronicotinamide were studied with the aid of double-irradiation decoupling techniques in a 100-Mcycle nuclear magnetic resonance spectrometer. The mechanism and kinetics of the primary protonation reactions of 1-methyl-, 1-propyl-, and 1-benzyldihydronicotinamides were also studied with the aid of nuclear magnetic resonance spectroscopy. It was shown that all signals due to ring protons decay simultaneously, following second-order kinetics. At the same time, new signals appear in the nuclear magnetic resonance spectra of these reaction mixtures. Assignments of chemical shifts for the new signals were achieved with the aid of 2-, 4-, and 6-monodeuterio derivatives of the above compounds and by means of kinetic considerations. The structure of the primary protonation product of these dihydropyridine derivatives, as emerges from this study, is more or less consistent with concepts based on spectrophotometric studies.

The spontaneous reactions of reduced nicotinamide–adenine dinucleotide (NADH)[1] with orthophosphates and other acidic anions in neutral watery milieu were previously followed by ultraviolet spectroscopy and the results were analyzed kinetically (Alivisatos *et al.*, 1964, 1965). These spontaneous reaction sequences may be related, as models, to biological interactions occurring during the first step (references in Lehninger and Wadkins, 1962) of oxidative phosphorylation. In this context, it was of interest to explore the applicability of nulcear magnetic resonance spectroscopy to our studies. In the present communication we report results obtained by interaction of model 1-substituted 1,4-dihydronicotinamides (1-R-DHN) with phosphate and monochloroacetate. It is shown that the kinetics and the mechanism of these processes may be substantially clarified by this new approach.

Materials and Methods

1-Methylnicotinamide iodide was prepared according to Karrer *et al.* (1936). It was reduced with sodium dithionite to 1-methyl-1,4-dihydronicotinamide by the

* From the Division of Enzymology, The Chicago Medical School, University of Health Sciences, Chicago, Illinois 60612. *Received August 14, 1967.* This investigation was supported in part by grants from the National Science Foundation (NSF GB 4645), the U. S. Public Health Service (CA 10143), and the American Cancer Society, Inc. (P-424). A preliminary report of this work was presented at the 154th Meeting of the American Chemical Society (Choi and Alivisatos, 1967). Inquiries should be made to Dr. S. G. A. A.

† Postdoctoral trainee.

[1] Abbreviations: NADH, reduced nicotinamide–adenine dinucleotide; 1-R-DHN, variously substituted (at position 1) 1,4-dihydronicotinamides.