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Edward

VIOMYCIN: THE STRUCTURE OF VIOMYCIDINE

A THESIS

Presented to  
The Faculty of the Graduate Division

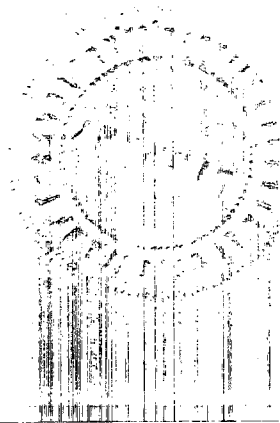
by

Edward Gifford Miller, Jr.

In Partial Fulfillment  
of the Requirements for the Degree  
Doctor of Philosophy  
in the School of Chemistry

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VIOMYCIN: THE STRUCTURE OF VIOMYCIDINE

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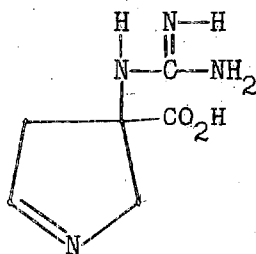
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## SUMMARY

The purpose of this research was to establish the correct structure for viomycidine, an hydrolysis product of the antibiotic viomycin. Viomycidine had been previously reported to have the formula  $C_6H_{10}N_4O_2$  and to contain no  $O-CH_3$ ,  $N-CH_3$ , or primary amino groups. At the beginning of this research 4-guanidino- $\Delta^1$ -pyrroline-4-carboxylic acid (I)



I

was suggested for the structure of viomycidine on the basis of the formula, the optical activity, the  $pK_a$  values, the presence of a mono-substituted guanidine group (Sakaguchi test), the nuclear magnetic resonance spectrum and the presence of a reducible double bond. Many of the previously reported experiments were repeated and new experiments were designed to gain evidence for or against the proposed structure for viomycidine.

The presence of a carboxylic acid group in viomycidine is shown by the  $pK_a$  value of 2.8 in 66 per cent dimethylformamide. The presence of a guanidine group in viomycidine is shown by the  $pK_a$  value of 13.4 in 66 per cent dimethylformamide. Viomycidine gives a positive test with

the Sakaguchi reagent indicating the presence of a mono-substituted guanidine group; the Sakaguchi reagent is specific for mono-substituted guanidines. The weakly basic group in viomycinine (pKa, 5.50 in water) exhibits a differential ultraviolet spectrum; this behavior is typical of tertiary amines but is not shown by primary and secondary amines.

Viomycinine and several known  $\Delta^1$ -pyrrolines were found to give stable yellow colors with *o*-aminobenzaldehyde; the only aliphatic imine tested gave a yellow color which faded rapidly. The ultraviolet and visible spectra of the yellow compounds resulting from three known  $\Delta^1$ -pyrrolines and viomycinine with *o*-aminobenzaldehyde were recorded. The spectra of the yellow compound from viomycinine is quite similar to the spectra of the yellow compound from the known  $\Delta^1$ -pyrrolines. This is strong evidence that there is a  $\Delta^1$ -pyrroline moiety in viomycinine. The recorded stability of  $\Delta^1$ -pyrrolines to hot, acidic conditions indicates that a  $\Delta^1$ -pyrroline is not excluded from being present in viomycinine. The pKa value of 5.50 in viomycinine was found to be consistent with a  $\Delta^1$ -pyrroline since known  $\Delta^1$ -pyrrolines have pKa values of 5.5 to 7.8.

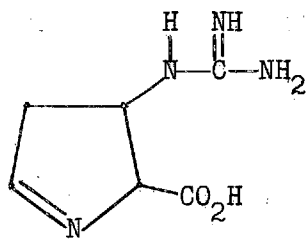
On the basis of I for viomycinine it was proposed that oxidation of viomycinine followed by acid-catalyzed decarboxylation should result in the formation of guanidinosuccinic acid. An authentic sample of guanidinosuccinic acid was prepared for comparison purposes. Several oxidation reagents, varying excesses of oxidant, different temperatures and various reaction times were used, but none of the reactions produced guanidinosuccinic acid. This is taken as evidence against structure I for viomycinine.

Since there is precedent for the reaction of  $\Delta^1$ -pyrrolines with acetic anhydride to form N-acetyl- $\Delta^2$ -pyrrolines, it was thought that an acetyl derivative of viomycidine could be prepared. An acetyl derivative of viomycidine was prepared whose formula ( $C_8H_{12}O_3N_4$ , derived from elemental analyses) was consistent with 1-acetyl-4-guanidino- $\Delta^2$ -pyrroline-4-carboxylic acid. Acetylviomycidine was treated with ozone. The ozonide was treated with hot, acidic hydrogen peroxide to decompose the ozonide, hydrolyze the acetyl group, and decarboxylate the malonic acid derivative. It was anticipated that barium hydroxide hydrolysis of the intermediate  $\alpha$ -guanidino- $\beta$ -aminopropionic acid would give  $\alpha,\beta$ -diaminopropionic acid. However, no  $\alpha,\beta$ -diaminopropionic acid was detectable.

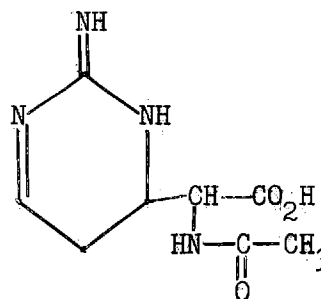
As a consequence of the attempted synthesis of dihydroviomycidine, a new amino acid, 3-aminopyrrolidine-3-carboxylic acid was prepared. This amino acid was characterized by the N,N'-dibenzoyl derivative and the di-p-hydroxyazobenzene-p'-sulfonate salt.

Viomycidine has been previously reported to evolve three equivalents of base on barium hydroxide hydrolysis. A mechanism for the evolution of this base, as ammonia, and for the simultaneous formation of pyrrole-3-carboxylic acid is proposed. A pyrrole-acid was obtained in 22% yield by barium hydroxide hydrolysis of viomycidine; this substance was conclusively identified as pyrrole-2-carboxylic acid. This strongly indicates that there are five carbon atoms in a straight chain in viomycidine, and excludes I as the structure of viomycidine. The formation of pyrrole-2-carboxylic acid is readily rationalized if viomycidine has the structure 4-guanido- $\Delta^1$ -pyrroline-5-carboxylic acid (II). The formation of 2-aminopyrimidine when viomycidine is hydrolyzed in concentrated

sodium hydroxide is also readily rationalized by II. From the proposed mechanism for the formation of 2-aminopyrimidine it would be expected that glycine would be formed. It was shown that glycine was produced, thus adding support to structure II for viomycin. Acetylviomycin gives a negative test with the Sakaguchi reagent, indicating that it does not contain a mono-substituted guanidine. The nuclear magnetic resonance spectrum,  $pK_a$  values, and other properties of acetylviomycin appear to be consistent with its formulation as III.



II

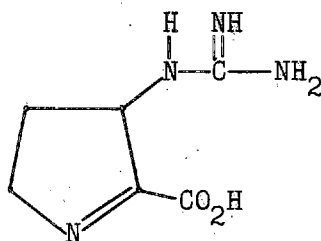


III

The nuclear magnetic resonance spectra of viomycin in deuterium oxide solution and trifluoroacetic acid solution are examined in detail. There are five exchangeable protons in viomycin and five carbon-bonded protons in a ratio of 2:2:1. The absorption at highest field was split into a triplet ( $J = 1.9$  cps.) if the deuterium oxide solution was deoxygenated. In trifluoroacetic acid solution viomycin shows three peaks in addition to the peaks observed in deuterium oxide solution. The protons in these peaks are in a ratio of 2:1:1. This is in contrast to the normal behavior observed with aliphatic guanido-acids such as  $\alpha$ -guanidopropionic acid and  $\beta$ -guanidopropionic acid. The number of peaks is interpreted in terms of a rigid hydrogen-bonded system.

Using known compounds ( $\Delta^1$ -pyrroline, 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid, proline, and pyrrolidine), the peak positions for structure II are estimated. All of the estimated values for structure II are in reasonable agreement with the absorption values observed for viomycin except the estimated value for the proton at the 2 position. Known  $\Delta^1$ -pyrrolines which are not substituted in the 2 position have an absorption at a  $\tau$  value of about 2. The lowest carbon-bonded proton absorption in viomycin is 4.07  $\tau$  in trifluoroacetic acid solution. This indicates that viomycin does not have an olefinic-type proton and, therefore, structure II does not explain all of the data observed for viomycin.

Structure IV (3-guanido- $\Delta^1$ -pyrroline-2-carboxylic acid) is proposed for viomycin. The estimated nuclear magnetic resonance absorption positions for IV are in much better agreement with the observed positions in viomycin than are the estimated values for II. The formation of pyrrole-2-carboxylic acid, 2-aminopyrimidine, and glycine are explained on the basis of a base catalyzed isomerization of IV to II.



IV

## INTRODUCTION

Viomycin.-- Viomycin is a tuberculostatic antibiotic which was isolated from cultures of Streptomyces puniceus and Streptomyces floridiae in 1950 in the laboratories of Charles Pfizer and Company (1) and Parke, Davis and Company (2). Although biological tests indicated that viomycin might be useful against tuberculosis (1), later studies on humans in the advanced stages of tuberculosis revealed several toxic side effects (3). Viomycin is still used clinically, especially when the tuberculosis micro-organism has become resistant to streptomycin. Ordinarily its use is limited to advanced cases of the disease for short periods of time.

Several salts of viomycin have been prepared; the sulfate melts at  $252^{\circ}$  with decomposition and has a specific rotation of  $-39.8^{\circ}$  (2). Diffusion molecular weight studies and elemental analyses resulted in a suggested formula of  $C_{25}H_{40-48}N_{12}O_{11} \cdot 3/2 H_2SO_4$  (4).

Positive Sakaguchi, ninhydrin and biuret tests indicated that viomycin contained guanidine and peptide groups (2). The antibiotic is a strong base, with  $pK_a^*$  values of 8.3, 10.3 and 12 in water. The  $pK_a$  of 12 is probably due to a guanidine group; those of 10.3 and 8.3 are probably due to amino functions. Absence of a low  $pK_a$  value indicates that viomycin probably does not have a free carboxyl group (4).

---

\*  $pK_a = -\log \frac{[H^+][A^n]}{[HA^{n+1}]}$ , where either  $A^n$  or  $HA^{n+1}$  can be the compound in question or the compound in a different state of protonation.

When hydrolyzed in 6 N hydrochloric acid at 100° viomycin yields ammonia, carbon dioxide, urea, L-β-lysine, L-serine, L-α,β-diaminopropionic acid and a mixture of compounds, each containing the guanidine group. One of these is produced in reasonable amounts (5) and has been named viomycidine.

Viomycidine--- Earlier workers separated the substances that gave positive Sakaguchi tests from the other products of acid hydrolysis of viomycin by ion-exchange chromatography. Further ion-exchange chromatography resulted in a preparation that was reasonably homogeneous but was not crystalline (2,4).

Analyses of the flavinate and p-hydroxyazobenzene-p'-sulfonate salts of this compound resulted in suggested formulas of  $C_6H_{16}N_4O_5$  and  $C_6H_{12}N_4O_3$  respectively for the free guanidine-containing compound. The compound was found to have a specific rotation in water of -63° and to have pKa values of 1.5, 5.7 and 12.4 in water. The compound gave a pink color with the Sakaguchi reagent, indicative of a mono-substituted guanidine function, a pink color with the ninhydrin reagent, and did not give a positive Tollen's test. The compound gave no color change with the Benedict-Behre reagent. When heated under the conditions for the transformation of creatine into creatinine, it did not give a positive test with the Benedict-Behre reagent and still gave a positive test with the Sakaguchi reagent. This indicated that no cyclization to give the creatinine-type linkage had occurred. It was found that the compound reduced 0.26 mole of periodate in 30 minutes and 1.05 mole in six hours at pH 8.4, indicating the probable absence of vicinal hydroxyl and/or

amino groups (4).

More recently, the major guanidine-containing compound (viomycin) has been isolated in crystalline form in an overall yield from viomycin of about 10 per cent. This isolation was accomplished by the use of ion-exchange resins and carbon chromatography (6).

Viomycin hydrochloride is a white crystalline solid which has the formula  $C_6H_{10}O_2N_4 \cdot HCl$ ; it melts with decomposition from 200 to 208°. It has no C-methyl, O-methyl, N-methyl or primary amino groups. The specific rotation varies with pH, being -21.2° in 2 N hydrochloric acid, -83.2° in water and -155.8° in 1 N sodium hydroxide (6).

Potentiometric titration data reveal that viomycin has  $pK_a$  values of 2.8, 5.87, and 13.4 in 66 per cent N,N-dimethylformamide and 5.50 and 12.6 in water (the carboxylic acid function was too strong to be accurately determined in water by the method used. Only end absorption is shown in the ultraviolet region. However, viomycin does exhibit a differential ultraviolet spectrum. A determination using pH 9.80 in one cell and pH 3.82 in the other shows that the weakly basic group absorbs at 212 m $\mu$  with an extinction coefficient of 2,530. This group is probably a tertiary amine of some kind since primary and secondary amines do not give a differential ultraviolet spectrum (6,7).

The infrared spectrum of viomycin hydrochloride shows a large number of absorption maxima. The only peaks to which structural features were assigned are those for N-H stretching, carboxylate anion vibration, C=N stretching, C-H stretching and  $CH_2$  deformation vibrations (6).

The nuclear magnetic resonance spectrum of viomycin hydrochloride



in deuterium oxide reveals that six of the eleven protons are exchanged for deuterium atoms. The remaining five protons are in a ratio of 1:2:2 and absorb at  $4.37\tau$ ,  $5.38\tau$  and  $7.43\tau$  respectively. The peak at  $5.38\tau$  appears to be a singlet; the peaks at  $4.37\tau$  and  $7.43\tau$  are each poorly resolved multiplets. The peak at  $4.37\tau$  is in the region where olefinic protons absorb (6).

With ninhydrin spray reagent on paper chromatograms viomycinine gives a pink-purple color, which is slightly weaker than that found for normal  $\alpha$ -amino acids. The Weber reagent produces a pink color with viomycinine, indicating either a mono-substituted or N,N'-di-substituted guanidine. The pink color with the Sakaguchi reagent indicates a mono-substituted guanidine. A negative ferric chloride test indicates the absence of an enolic group (6).

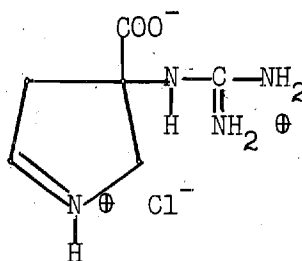
Barium hydroxide hydrolysis of viomycinine at  $90^\circ$  results in the evolution of 2.71 mole of volatile base and the formation of 0.45 mole of barium carbonate. A complex mixture of eight substances which gave positive ninhydrin tests was revealed by paper chromatography. Hydrolysis of viomycinine with sodium hydroxide solution at  $160-310^\circ$  results in the evolution of 3.75 mole of volatile base (6). Hydrolysis in concentrated sodium hydroxide at  $80^\circ$  produces a small amount of a sublimate which was identified as 2-aminopyrimidine (8).

Heating with concentrated hydrochloric acid for six days resulted in the incomplete conversion of viomycinine into at least five ninhydrin-positive compounds, some of which were Weber-negative. An appreciable amount of viomycinine still remained after this length of time (6).

Possibly, this accounts for the low yield of even the major guanidine compound as well as for the number of guanidine compounds produced in the hydrolysis of viomycin. The other guanidine compounds formed from viomycin have not been isolated in pure form.

Hydrogenation of viomycinidine with acetic acid-water as the solvent and platinum on carbon as the catalyst results in the absorption of one mole of hydrogen. No pure hydrogenation product was isolated (6).

At the beginning of this research the structure 4-guanido- $\Delta^1$ -pyrroline-4-carboxylic acid hydrochloride (I) was suggested for viomycinidine hydrochloride (8).



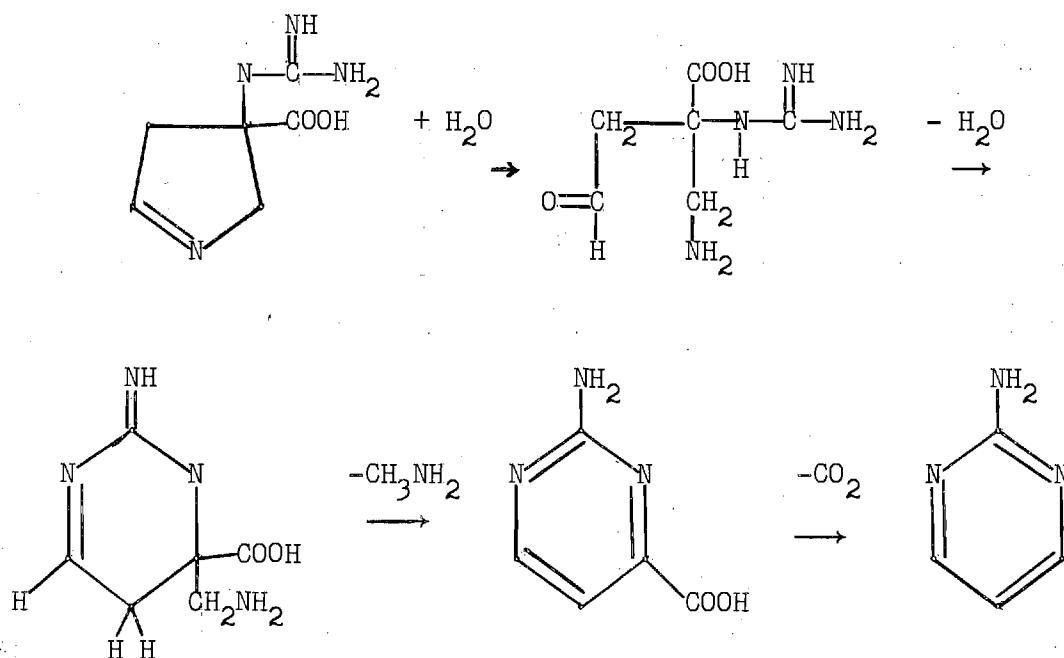
I

The facts which lead to this structure may be enumerated as follows:

- (1) The analytical data. (2) Viomycinidine is optically active, indicating at least one asymmetric carbon atom. (3) The  $pK_a$  value of 2.8 indicates a carboxylic acid group; the value of 13.4 indicates a guanidine group; the value of 5.87 indicates a weakly basic amine and the differential ultraviolet spectrum further indicates that the amine is tertiary.
- (4) The positive Sakaguchi test indicates that a mono-substituted guanidine function is present. (5) The nuclear magnetic resonance spectrum indicates one methylene group not adjacent to a carbon atom bearing

a proton. The other two peaks are split and therefore must be adjacent to each other. Further, the peak containing only one proton is in the olefinic region. Thus, the part structure  $-\text{CH}_2-$  and  $-\text{CH}_2-\overset{\text{H}}{\text{C}}=$  is indicated.

(6) Viomycidine takes up one mole of hydrogen under perhydrogenation conditions, substantiating the proposal that an olefinic linkage is present and indicating that the compound has one ring. One carbon atom of viomycidine is present in a carboxyl group and another is contained in the guanidine group. The nature of three of the remaining four carbon atoms is indicated by the nuclear magnetic resonance spectrum. The remaining carbon atom can bear no proton, must be the asymmetric atom of viomycidine and, furthermore, must bear both carboxyl and guanidine substituent groups. All of the evidence is readily explained by structure I. The following proposed pathway from I to 2-aminopyrimidine further supports the proposed structure (8).



The purpose of this research was to gain chemical evidence for structure I or to elucidate the correct structure for viomycin.

## EXPERIMENTAL

Apparatus and Techniques.-- Ion exchange resins used in this work were regenerated and used as described previously (6). The following is a list of these resins and the abbreviations used for each throughout the rest of this thesis: Amberlite anion exchange resin 45 in either the hydroxyl or chloride phase, IRA 45 ( $\text{OH}^-$ ) or IRA 45 ( $\text{Cl}^-$ ); Amberlite anion exchange resin 400 in the hydroxyl phase, IRA 400 ( $\text{OH}^-$ ); Amberlite cation exchange resin 50 in the hydrogen phase, IRC 50 ( $\text{H}^+$ ). When ion exchange resins were used batchwise or in columns, a minimum of a five-fold excess of resin was used. When resins were used in columns the sample was eluted with a minimum of two column-volumes of eluting solvent. When resins were used batchwise, the sample was eluted by washing the resin with about four times as much water as the quantity of resin used.

The apparatus and techniques used in paper chromatography were the same as those described previously (6). The solvent systems which were most frequently used and their abbreviations used in the balance of this thesis are: t-butyl alcohol-acetic acid-water, 2:1:1 (v/v) (BAW); n-propyl alcohol-acetic acid-water, 10:1:9 (v/v) (PAW); phenol-water, 4:1 (v/v) (PW). Spray reagents which were most frequently used were ninhydrin and Weber. Their preparation and interpretation are given in reference 9.

The following qualitative color tests were used and the method for

performing the test is given in the reference cited for each test: ninhydrin (9), Weber (9), Sakaguchi (9), Nessler (10), Erhlich (11), ferric chloride (12), a p-dimethylaminobenzaldehyde reagent for urea compounds (9), and Benedict-Behre (13).

Pretreated Darco G-60 (Atlas Powder Company) was prepared and mixed with acid-washed Celite (Johns-Manville Corporation) as described previously (6). The carbon columns so prepared were washed with tap water until the wash water gave a negative test for alcohol with the ceric nitrate reagent (14).

Routinely, 100 g. of charcoal-celite mixture was used per gram of sample being chromatographed. The cellulose columns were prepared as described previously (6).

All melting points were determined on a Kofler micro hot stage. All optical rotations were determined with a Bellingham and Stanley Ltd. polarimeter, using the D line of sodium as a light source. All infrared spectra were determined on a Perkin Elmer Model 137 recording spectrophotometer. The ultraviolet spectra were determined on either a Beckman Model Dk-1 recording spectrophotometer, a Bausch and Lomb Model 505 recording spectrophotometer or a Cary Model 14 recording spectrophotometer.

The nuclear magnetic resonance spectra were determined on a Varian Model A-60 spectrometer. Reference compounds used and their abbreviations are: tetramethylsilane internal standard (TMS), tetramethylsilane (5% in carbon tetrachloride) external standard (TMS, external standard), and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The latter compound was prepared according to Tiers (15). The fraction collector which was used in the course of all column chromatographic separations

was a Research Specialties Company Model 1205. Microanalyses were performed by Galbraith Laboratories (Knoxville, Tennessee) and Huffman Laboratories (Wheatridge, Colorado).

Preparation of Viomycin Hydrochloride.-- Viomycin hydrochloride was isolated and purified by the method of Hayes (6). In the first preparation 165 g. of a mixture of crude viomycin sulfate and crude viomycin hydrochloride was used. After acid hydrolysis, the acidic solution was neutralized with IRA 45 ( $\text{OH}^-$ ) batchwise and passed over an IRA 400 ( $\text{OH}^-$ ) column. The eluate from this column was passed directly over an IRC 50 ( $\text{H}^+$ ) column. The IRC 50 ( $\text{H}^+$ ) column was washed with water and the basic material was eluted with 1 N hydrochloric acid. The acidic eluate was neutralized with IRA 45 ( $\text{OH}^-$ ). The resin was removed by filtration and the filtrate was passed over an IRA 45 ( $\text{Cl}^-$ ) column. The eluate was evaporated to dryness in vacuo to give 42.3 g. of basic material. This material was chromatographed on a charcoal column. The first fractions consisted primarily of ammonium chloride and weighed 22.2 g. The fractions which contained viomycin hydrochloride as the major material weighed 7.5 g. These were combined and crystallized to give 3.12 g. of pure viomycin hydrochloride, m. p.  $201-208^\circ$  dec. (corr.) (lit.  $200-210^\circ$  (6)),  $[\alpha]_D^{27} = -86^\circ$  (c 2.24, water) (lit.,  $[\alpha]_D = -83.^\circ$  (c 1.68, water) (6)).

In the second preparation an amount of viomycin sulfate (Parke, Davis and Company, lot No. H19606A) was used which was equivalent to 20 g. of viomycin base. The purification was as described above except that the IRA 400 column was eluted with about 15 column-volumes of water

rather than three column-volumes as used by Hayes. The weight of the strongly basic material obtained by ion-exchange treatment of viomycin hydrolysate was 7.8 g. This material was chromatographed on a charcoal column and paper chromatograms were run on pooled fractions from this column. The first pooled fraction after ammonium chloride (2.9 g.) weighed 1.04 g. and was found to consist of viomycin hydrochloride and a compound with a lower  $R_F$  value than viomycin hydrochloride. In BAW, viomycin hydrochloride had an  $R_F$  value of 0.28 and the other compound had an  $R_F$  value 0.13. This compound gave a pink color with Weber reagent and a green-gray color with ninhydrin reagent. The intensity of the Weber color was about the same for the lower  $R_F$  compound and viomycin hydrochloride. This fraction was set aside for future work. The remaining fractions from the charcoal column weighed 2.2 g. and were crystallized three times to give 0.62 g. (10 per cent) of pure viomycin hydrochloride, m. p. 203-210° dec. (corr.).

Both preparations of viomycin hydrochloride gave the same  $R_F$  value in BAW, PAW, and PW as a sample of viomycin hydrochloride which had been prepared by Hayes. Viomycin hydrochloride was found to give two spots in the solvent system PW with  $R_F$  values of 0.23 and 0.76 when sprayed with ninhydrin and Weber reagents. A solution of 26 mg. of viomycin hydrochloride in 0.2 ml. of water was applied to thick chromatography paper (Whatman No. 17) 25 cm. wide; the chromatogram was run in PW. The high and low  $R_F$  spots were eluted separately from the paper with water and the two eluates were concentrated to about 0.5 ml. Paper chromatograms were run on each eluate in PW and sprayed with Weber and ninhydrin reagents. From each eluate there was observed two spots of



$R_F$  0.23 and 0.76.

About 0.2 mg. of viomycin hydrochloride was heated at  $100^\circ$  with 1 ml. of 0.2 per cent ninhydrin in water-saturated 1-butanol. After three minutes a pink color appeared which was only slightly weaker than with the normal ninhydrin reagent.

The nuclear magnetic resonance spectrum of viomycin hydrochloride was determined using a deuterium oxide solution (DSS) ( $\approx 21.2\%$ ) and showed peaks at  $7.43 \tau$  (2 H, poorly resolved multiplet),  $5.38 \tau$  (2 H, singlet) and  $4.37 \tau$  (1 H, poorly resolved multiplet). The number of protons in each peak was determined by using the automatic integrator. The nuclear magnetic resonance spectrum of viomycin hydrochloride in trifluoroacetic acid solution (TMS) ( $\approx 10\%$ ) showed peaks at  $7.23 \tau$  (2 H, singlet),  $5.05 \tau$  (2 H, singlet),  $4.07 \tau$  (1 H, singlet),  $3.00 \tau$  (2 H, singlet),  $1.98 \tau$  (1 H, singlet) and  $1.48 \tau$  (1 H, singlet). The number of protons was determined by cutting out and weighing the paper enclosed by each peak. The spectrum is given as Figure 1.

In another experiment the nuclear magnetic resonance spectrum of a deoxygenated solution of viomycin hydrochloride in deuterium oxide ( $\approx 10\%$ ) (TMS, external standard) was recorded. The number of protons was determined by cutting out and weighing the paper enclosed by each peak. In one spectrum the amplitude was adjusted so that the water peak was on scale. The water peak from the deuterium oxide used was subtracted from the water peak observed in the spectrum of viomycin hydrochloride. Peaks were observed at  $7.43 \tau$  (2.00 H, triplet,  $J = 1.9$  cps.),  $5.38 \tau$  (1.96 H, singlet),  $5.17 \tau$  (5.95 H, singlet), and  $4.37 \tau$  (1.11 H, poorly resolved multiplet). This spectrum is given in Figure 2. A spectrum of

this solution was also run at a higher amplitude, so that the water peak was off scale and the other peaks were larger. In this way peaks were observed at 7.43  $\tau$  (2.00 H, triplet,  $J = 1.9$  cps.), 5.38  $\tau$  (2.05 H, singlet), and 4.37  $\tau$  (1.04 H, poorly resolved multiplet).

A nuclear magnetic resonance spectrum of a deoxygenated solution of viomycinidine hydrochloride in water was determined (c 10%) (TMS, external standard). It showed peaks at 7.43  $\tau$  (2 H, triplet,  $J = 1.9$  cps.), 5.37  $\tau$  (2 H, singlet), 4.37  $\tau$  (1 H, poorly resolved multiplet) and 2.57  $\tau$  (1.1 H, broad singlet). The number of protons in the peak with a 2.57  $\tau$  value was determined by tracing this peak and the peak with a 7.43  $\tau$  value and cutting out and weighing the paper enclosed by each peak. The value for the peak with a 7.43  $\tau$  value was set equal to two protons.

An ultraviolet spectrum of a solution of viomycinidine hydrochloride in water showed  $\epsilon$ , 30 at 230  $m\mu$  and  $\epsilon$ , 1,200 at 210  $m\mu$ .

Ultraviolet spectra of viomycinidine hydrochloride in a solution of pH 3.57 (saturated solution of potassium bitartrate) (c 0.0058) and in a solution of pH 9.00 (Coleman buffer tablet) (c 0.0058) were recorded. The spectra of the solution of lower pH was subtracted from the spectrum of the solution of higher pH. When this was done, viomycinidine showed  $\lambda_{\max}$ . 210  $m\mu$ ,  $\epsilon$ , 2,400.

In the same way, the ultra-violet spectra of triethylamine, di-n-propylamine and n-propylamine were recorded in solutions of pH 9 (Coleman buffer tablet) and pH 13 (sodium hydroxide solution). The spectrum from the solution of lower pH was subtracted from the spectrum from the

solution of higher pH. None of the spectra showed a  $\lambda_{\text{max}}$ . but, at 210 m $\mu$ , triethylamine showed  $\epsilon$ , 3,000, di-n-propylamine showed  $\epsilon$ , 400, and n-propylamine showed  $\epsilon$ , 80. The light path for all of these spectra was 1 mm.; the spectra were recorded on the Cary Model 14 spectrophotometer.

Acetylviomycin. --- To a solution of 100 mg. (0.48 mmole) of viomycin hydrochloride in 3 ml. of water was added enough absolute ethanol to furnish a cloudy solution at 10°; there was then added 3 ml. (30 mmoles) of acetic anhydride. The solution was allowed to stand in the refrigerator for two days. The acidic solution was then treated batchwise with IRA 45 (OH<sup>-</sup>) to neutralize acetic acid. After filtration the solution was evaporated to about 10 ml. at 50° in vacuo; the solution was then lyophilized. About 3 ml. of water was added and again the solution was lyophilized; this was repeated two times. The resulting tan solid was dissolved in 3 ml. of 95 per cent ethanol and centrifuged. The supernatant solution was evaporated on the steam bath to 1 ml., whereupon a white solid precipitated. This was collected, air dried, and found to weigh 50 mg. (48%). This was crystallized from 9.0 ml. of 88 per cent acetone-water giving 40 mg. of white crystalline solid, m.p. 256-257° dec. (corr.),  $[\alpha]_{\text{D}}^{28} = +41^{\circ} \pm 1^{\circ}$  (c 2.4, water).

Anal.  $\text{C}_8\text{H}_{12}\text{N}_4\text{O}_3$   
(212.24)

Calc'd: C, 45.28; H, 5.70; N, 26.43  
Found : C, 45.41; H, 5.91; N, 26.59

The  $R_F$  values (Weber) for acetylviomycin were: 0.57 (BAW, orange), 0.67 (PAW, orange) and 0.80 (PW, orange). The ultraviolet spectrum in 95% ethanol showed no absorption peak but only end absorption;

$\epsilon$ , 50 at 230  $m\mu$ ,  $\epsilon$ , 7,500 at 210  $m\mu$  and  $\epsilon$ , 10,000 at 200  $m\mu$ . 2-Butenoic acid, in 95 per cent ethanol, had  $\lambda_{\max}$ . 206  $m\mu$ ,  $\epsilon$ , 10,300 (lit.  $\lambda_{\max}$ . 204  $m\mu$ ,  $\epsilon$ , 11,700 (16)). The infrared spectrum of acetylviomycinidine in a potassium bromide pellet, is given in Figure 3. Acetylviomycinidine had  $pK_a$  values of 4.86 and 13.0 in 66 per cent dimethylformamide as shown by potentiometric titration (34).

The nuclear magnetic resonance spectrum was recorded in deuterium-oxide solution (TMS, external standard) (c 10%) (Figure 4) and showed peaks at 4.30  $\tau$  (0.784 H, poorly resolved multiplet), 5.19 $\tau$  (4.26 H, singlet), 5.50  $\tau$  (1.96 H, poorly resolved multiplet), 7.73  $\tau$  (3.44 H, singlet), and 8.03  $\tau$  (1.57 H, singlet). The number of protons in each peak was determined by automatically integrating the spectrum, taking the total height for all peaks and dividing by 12, the total number of protons present in acetylviomycinidine. The peaks were run at a higher amplitude, traced, and the tracing cut out and weighed. The peaks at 5.50  $\tau$  and 4.30  $\tau$  were in a ratio of 2.00:1.03; the peaks at 7.33  $\tau$  and 8.03  $\tau$  were in a ratio of 3.00:1.22 (or 3.44:1.40).

Acetylviomycinidine did not give a color with the ninhydrin reagent or with the  $\alpha$ -aminobenzaldehyde reagent. A solution of acetylviomycinidine in water failed to give a precipitate on adding silver nitrate solution. Acetylviomycinidine gave a negative test for a mono-substituted guanidine with the Sakaguchi reagent and a negative test for the creatinine-type linkage with the Benedict-Behre reagent.

About 0.5 mg. of acetylviomycinidine was dissolved in four drops of 6 N hydrochloric acid and the solution was heated at 100° for two hours. Paper chromatograms of the acetylviomycinidine hydrolysate and viomycinidine

were run in BAW and PAW. The hydrolysate showed one spot in each system with  $R_F$  values of 0.28 and 0.53 respectively. The values were the same as for viomycin. The chromatograms were sprayed with ninhydrin and Weber reagents and the colors were the same for the hydrolysate and for viomycin.

2,4-Dinitrophenylviomycin.--- The method used for the preparation of 2,4-dinitrophenylviomycin is an adaption of a general method for the preparation of 2,4-dinitrophenylamino acids (17). At room temperature, 50 mg. (0.24 mmole) of viomycin hydrochloride was added to 3 ml. of water which contained 0.25 g. of sodium bicarbonate. To this solution was added a solution of 0.28 g. (1.5 mmole) of 2,4-dinitrofluorobenzene in 7 ml. of absolute ethanol. The suspension was stirred magnetically for two hours at room temperature and then refrigerated overnight. The reaction mixture was acidified and after extraction with benzene to remove 2,4-dinitrophenol and 2,4-dinitrofluorobenzene, the solution was neutralized with ammonium hydroxide. The product crystallized and was collected. It was crystallized twice from water and dried for 12 hours at 78° and 0.2 mm., giving 20 mg. (21%) of yellow needles, m. p. 171.5-172.5° (corr.).

<u>Anal.</u> $C_{12}H_{12}O_6N_6 \cdot 2 H_2O$	Calc'd: C, 38.71; H, 4.33; N, 22.58
(372.20)	Found : C, 38.71; H, 4.57; N, 23.08

2,4-Dinitrophenylviomycin gave a pink color with Weber reagent as did 2,4-dinitrophenylarginine. 2,4-Dinitrophenylviomycin had  $R_F$  values (yellow color, no spray) of 0.70, 0.85 and 0.55 in BAW, PAW and PW respectively. The infrared spectrum was taken in a potassium bromide

pellet and is given in Figure 5.

Iso-butyldeneethylamine.-- A sample of iso-butyldeneethylamine was prepared by the method of Campbell (18). The preparation was distilled, b.p. 87-88° (lit. (18) 90°/767 mm.). Nuclear magnetic resonance spectra of this compound were determined. As the neat liquid (TMS), the imine showed peaks at 2.47  $\tau$  (1 H, doublet,  $J = 3.8$  cps.), 6.70  $\tau$  (2 H, quartet,  $J = 7.2$  cps.), 7.70  $\tau$  (1 H, multiplet;), 8.93  $\tau$  (3 H, triplet,  $J = 8.2$  cps.), and 8.97  $\tau$  (6 H, doublet,  $J = 7.2$  cps.). In trifluoroacetic acid (TMS) (c 50%) the imine showed peaks at 1.66  $\tau$  (1 H, poorly resolved multiplet), 6.12  $\tau$  (2 H, quartet,  $J = 6.0$  cps.), 7.30  $\tau$  (1 H, poorly resolved multiplet), 8.53  $\tau$  (3 H, triplet,  $J = 7.0$  cps.), and 8.68  $\tau$  (6 H, doublet,  $J = 7.0$  cps.).

Di-iso-butyldeneazine.-- A sample of di-iso-butyldeneazine was prepared by the method of Malbe (19). The preparation was distilled, b.p. 164-165° (lit. (19) 160-165°). The nuclear magnetic resonance spectrum of the azine was obtained as the neat liquid (TMS) and showed peaks at 2.32  $\tau$  (2 H, doublet), 7.53  $\tau$  (2 H, sextet), and 8.93  $\tau$  (12 H, doublet).

Preparation and Properties of  $\Delta^1$ -Pyrroline.-- The mercuric chloride complex of  $\Delta^1$ -Pyrroline was prepared by the method of Skursky (20). To a solution of 5.74 g. (0.021 mole) of mercuric chloride and 4.54 g. (0.021 mole) of sodium periodate in 200 ml. of water was added, at 0°, 2.03 g. (0.011 mole) of proline. The solution was allowed to stand in the refrigerator for 12 hours. The resulting crystalline precipitate was collected and triturated with eight 25-ml. portions of ice water. The solid

was then washed with ethanol and dried in a desiccator, giving 4.5 g. (84%). The infrared spectrum of the compound was determined as a nujol mull and showed, among other absorptions, a strong sharp peak at  $6.12 \mu$ .  $\Delta'$ -Pyrroline had a  $pK_a$  value of 5.6 in water as shown by potentiometric titration (34).

The mercuric chloride complex of  $\Delta'$ -pyrroline (980 mg., 0.0029 mole) was slurried with 2.0 ml. of trifluoroacetic acid and the mixture was centrifuged. A nuclear magnetic resonance spectrum of the supernatant liquid (TMS) was immediately recorded and showed peaks at  $1.20 \tau$  (1 H, singlet),  $5.68 \tau$  (2 H, poorly resolved multiplet),  $6.67 \tau$  (2 H, poorly resolved multiplet), and  $7.47 \tau$  (2H, quintet,  $J = 7.6$  cps.); this spectrum is shown as Figure 7. A spectrum of the same solution one week later showed no change.

Hydrogen sulfide was passed into a solution of 5 g. (14.8 mmole) of the mercuric chloride complex of  $\Delta'$ -pyrroline in 140 ml. of 0.3 N hydrochloric acid for about 30 minutes. The resulting mixture was slurried with charcoal and celite and filtered through a celite pad on a sintered glass funnel. Excess hydrogen sulfide was removed from the filtrate in vacuo at  $40^\circ$ . IRA 45 ( $OH^-$ ) was added to the solution until the pH of the solution was 3.5. Over a period of about 30 minutes, 7.5 g. (175 mmole) of acetic anhydride and IRA 45 ( $OH^-$ ) were added to the solution such that the pH of the solution was maintained at  $3.5 \pm 0.1$ . The resin was removed by filtration and the filtrate was saturated with sodium chloride. The solution was extracted continuously with chloroform for 12 hours. The chloroform was dried over magnesium sulfate and evaporated in vacuo to give 150 mg. of red oil. An infrared spectrum of this oil showed a broad peak at  $6.07 \mu$ . A nuclear magnetic resonance spectrum of the chloroform

extract in carbon tetrachloride solution (TMS) showed weak absorption at 4.83  $\tau$  and strong absorptions at 6.58  $\tau$ , 8.13  $\tau$ , and 8.92  $\tau$ . All of the absorptions were broad and poorly resolved.

Properties of 2-Methyl- $\Delta^1$ -pyrroline-5-carboxylic Acid Hydrochloride.-- A sample of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid hydrochloride (21), m. p. 175-177° (lit. (22) 186-189°), gave a brown color with ninhydrin reagent in a test tube. The nuclear magnetic resonance spectrum of this  $\Delta^1$ -pyrroline in deuterium oxide solution (TMS, external standard) ( $\leq$  20%) was recorded and showed peaks at 7.36  $\tau$  (5 H, a singlet of 3 H superimposed on a multiplet of 2 H), 6.66  $\tau$  (2 H, triplet,  $J = 7.8$  cps.), and 4.76  $\tau$  (1 H, triplet,  $J = 9.0$  cps.). In trifluoroacetic acid solution (TMS) ( $\leq$  20%), the nuclear magnetic resonance spectrum of this  $\Delta^1$ -pyrroline showed peaks at 7.30  $\tau$  (5 H, broad), 6.58  $\tau$  (2 H, triplet,  $J = 8$  cps.) and 4.60  $\tau$  (1 H, triplet,  $J = 9.5$  cps.). This  $\Delta^1$ -pyrroline in water had  $pK_a$  values of 2.0 and 7.37 as shown by potentiometric titration (34).

To a solution of 300 mg. (1.8 mmole) of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid hydrochloride and 1.5 g. of sodium bicarbonate in 18 ml. of water was added a solution of 1.48 g. (7.9 mmole) of 2,4-dinitrofluorobenzene in 37 ml. of ethanol. The mixture was stirred magnetically for two hours at room temperature and placed in the refrigerator for 12 hours. The ethanol was evaporated in vacuo and the residue was acidified with 1.0 N hydrochloric acid. The resulting suspension was extracted with benzene to remove 2,4-dinitrophenol and excess 2,4-dinitrofluorobenzene. The aqueous suspension was then extracted with ethyl acetate. The ethyl acetate was dried over magnesium sulfate and evaporated to dryness in vacuo.



The resulting yellow solid was crystallized twice from 10 per cent acetone-benzene to give 200 mg. (35%) of yellow plates, m. p. 131-142° dec. (corr.). The derivative was crystallized three times for analysis from 50 per cent aqueous-ethanol, m. p. 132-142° dec. (corr.). The analytical sample was dried to constant weight at the analytical laboratory. An infrared spectrum of the derivative, in a potassium bromide pellet, showed, among others, absorptions at 2.96  $\mu$ , 5.79  $\mu$ , and 5.93  $\mu$ .

<u>Anal.</u> $C_{12}H_{11}N_3O_6$	Calc'd: C, 49.14; H, 3.88; N, 14.33
$C_{12}H_{11}N_3O_6 \cdot H_2O$	Calc'd: C, 46.30; H, 4.21; N, 13.50
(311.35)	Found : C, 46.02; H, 4.54; N, 12.93

To a solution of 100 mg. (0.61 mmole) of 2-methyl- $\Delta'$ -pyrroline-5-carboxylic acid hydrochloride in 3 ml. of water was added 7 ml. of ethanol at 0°. To this solution 3 ml. of acetic anhydride was added and the solution was allowed to stand in the refrigerator for two days. The ethanol was evaporated in vacuo and the residue lyophilized from water eight times to give a white solid. This solid was crystallized from 70 per cent acetone-methanol to give 55 mg. of white crystals, m. p. 170-177° dec. (corr.). No depression of melting point was observed on mixed melting with starting material.

o-Aminobenzaldehyde Reagent.--- The o-aminobenzaldehyde reagent was prepared by the method of Jakoby and Fredericks (23). The reagent contained about 40 mg. of o-aminobenzaldehyde in 10 ml. of water. When used as a qualitative color test, 5 mg. of the compound to be tested, 0.5 ml. of 0.025 N hydrochloric acid and 0.5 ml. of the reagent were mixed and heated on a steam bath for about two minutes. A yellow color

constituted a positive test. In this way, viomycidine, the mercuric chloride complex of  $\Delta'$ -pyrroline, 2,3-dimethyl- $\Delta'$ -pyrroline-5-carboxylic acid hydrochloride (21), 2-methyl- $\Delta'$ -pyrroline-5-carboxylic acid hydrochloride (21), 2-ethyl-3,3-diphenyl- $\Delta'$ -pyrroline (24), 2-methyl-3,3-diphenyl- $\Delta'$ -pyrroline (24), 4,4,5-triphenyl- $\Delta'$ -pyrroline (24), 4,4-diphenyl-5-(p-tolyl)- $\Delta'$ -pyrroline (24) and iso-butylidinediethylamine gave positive tests. o-Aminobenzaldehyde, acetamidine, guanidoacetic acid, glycine and viomycin gave negative tests.

In semi-quantitative experiments, a mixture of 0.50 ml. of the o-aminobenzaldehyde reagent, 0.50 ml. of 0.025 N hydrochloric acid, and 0.05 mmole of the compound to be tested was heated at 90° for 30 minutes. The reaction mixture was diluted to 15 ml. with distilled water and the ultraviolet and visible spectra were recorded. Some of these data are recorded in Figure 6.

Hydrogenation of Viomycidine.-- A mixture of 30 mg. of viomycidine hydrochloride, 100 mg. of five per cent ruthenium on alumina and 5 ml. of 75 per cent acetic acid was stirred under hydrogen for 24 hours at room temperature and atmospheric pressure. At the end of this time 100 mg. of the same catalyst was added and hydrogenation was continued for another 24 hours. The mixture was filtered and the acetic acid was neutralized with IRA 45 (OH<sup>-</sup>). The resin was removed by filtration and the filtrate passed over an IRA 45 (Cl<sup>-</sup>) column. The eluate was evaporated to about 1 ml. in vacuo and paper chromatograms were run in BAW. A sample of viomycidine hydrochloride was run on the same sheet and had an R<sub>F</sub> value of 0.45. The chromatograms were sprayed with Weber and ninhydrin reagents. Three more 30 mg. samples of viomycidine hydrochloride were

hydrogenated in the same way using five per cent ruthenium on carbon, five per cent rhodium on alumina and five per cent rhodium on carbon as catalysts. The work up was the same for these three hydrogenations as for the first hydrogenation. All of the papergrams showed two spots of about equal intensity with  $R_F$  values of 0.51 and 0.45 except the one in which rhodium on carbon had been used. From this catalyst, only one spot of  $R_F$  0.51 was observed. Also, the solution from this hydrogenation was colorless while the other three were brownish-yellow in color.

A mixture of 206 mg. (1.0 mmole) of viomycinidene hydrochloride and 1.33 g. of five per cent rhodium on carbon in 40 ml. of 75 per cent acetic acid was hydrogenated for four days at room temperature and atmospheric pressure. At the end of this time, 26 ml. of hydrogen, at  $24^\circ$  and 740 mm. of pressure had been absorbed (23 ml. at S. T. P., 1.03 mmole). Hydrogenation was continued for an additional five days; about 2 ml. of hydrogen was absorbed per day and during the last day less than 1 ml. was absorbed. Hydrogenation was stopped. The total uptake of hydrogen was about 1.3 mole of hydrogen per mole of viomycinidene. The catalyst was removed by filtration and the acetic acid was neutralized with IRA 45 ( $\text{OH}^-$ ). The resin was removed by filtration and the filtrate was passed over an IRA 45 ( $\text{Cl}^-$ ) column. The eluate was evaporated to dryness in vacuo and about 200 mg. of brown solid was obtained. Paper chromatograms were run in BAW and sprayed with Weber and ninhydrin reagents. Only one spot was observed and it had the same  $R_F$  value and had the same color as viomycinidene. The optical rotation of the crude preparation of dihydroviomycinidene hydrochloride was taken and found to be:

$$[\alpha]_D^{28} = +8.6^\circ \pm 1.4^\circ (\text{c } 7.2, \text{ water}).$$

The crude preparation was dissolved in about 2 ml. of water and a saturated aqueous solution of p-hydroxyazobenzene-p'-sulfonic acid was added. Enough of this solution was added to give a very deep red solution which indicated an excess of the sulfonic acid. An orange-yellow solid separated and was collected by filtration. The amorphous solid was washed with cold water, dried under vacuum in a desiccator and found to weigh 197 mg. The filtrate was concentrated and a second crop obtained which was found to weigh 80 mg. A third crop was obtained by evaporating the filtrate and it was found to weigh 260 mg. All crops were amorphous and could not be obtained crystalline.

Crops one and two were combined, dissolved in water and treated batchwise with IRA 45 ( $\text{OH}^-$ ). The colorless filtrate was then passed over an IRA 45 ( $\text{Cl}^-$ ) column and the eluate evaporated to dryness in vacuo. About 60 mg. of brown solid remained. Crop three was treated in the same way to give 90 mg. of brown solid. All attempts to crystallize either crop failed. The two crops were combined and a nuclear magnetic resonance spectrum was recorded in deuterium oxide. No absorbance was present having a  $\tau$  value of less than 5.

Attempted Hydrogenation of Guanidoacetic Acid.-- A solution of 234 mg. (2.00 mmole) of guanidoacetic acid in 8 ml. of 50 per cent aqueous acetic acid was stirred under hydrogen at room temperature and atmospheric pressure with 500 mg. of five per cent rhodium on carbon. A hydrogen uptake of 2 ml. (0.008 mmole) could have been observed with the apparatus used. No measurable amount of hydrogen was absorbed during two days.

3-Aminopyrrolidine-3-carboxylic Acid Hydrochloride.-- 1-Carbethoxy-4-pyrrolidone was prepared by the method of Kuhn and Osswald (25). The preparation distilled at 77-83°/0.5 mm (Lit. (25) 122-132°/12 mm). A nuclear magnetic resonance spectrum of the neat liquid (TMS) showed peaks at 8.78  $\tau$  (3 H, triplet,  $J = 7.1$  cps.), 7.45  $\tau$  (2 H, triplet,  $J = 8.0$  cps.), 6.37  $\tau$  (2 H, singlet), 6.27  $\tau$  (2 H, triplet,  $J = 8$  cps.), and 5.95  $\tau$  (2 H, quartet,  $J = 7.9$  cps.).

Four identical mixtures were prepared which contained 372 mg. (2.37 mmole) of 1-carbethoxy-4-pyrrolidone, 308 mg. (4.74 mmole) of potassium cyanide, 250 mg. (4.74 mmole) of ammonium chloride and 1 ml. of 30 per cent aqueous ammonia (17.7 mmole) in 25 ml. of distilled methanol. After two days, the methanol was evaporated from one of the mixtures and about 10 ml. of water was added to the residue. The aqueous solution was extracted with chloroform and the chloroform was washed with 1 N hydrochloric acid. The acidic solution was made basic with potassium carbonate and extracted with chloroform. The chloroform was dried over magnesium sulfate and evaporated in vacuo to give 168 mg. of red-brown oil. This oil showed a weak absorption at 4.5  $\mu$ . The remaining reaction mixtures were allowed to react for four, six, and nine days. Each was treated in the same way as the two-day reaction. From the four-day reaction there was obtained 154 mg. of basic fraction, from the six-day reaction 232 mg. of basic fraction and from the nine-day reaction 260 mg. of basic fraction. Each basic fraction showed an absorption at 4.5  $\mu$ ; the intensity of this absorption was greatest for the nine-day reaction.

A mixture of 10.00 g. (0.064 mole) of 1-carbethoxy-4-pyrrolidone, 8.25 g. (0.128 mole) of potassium cyanide, 6.8 g. (0.128 mole) of ammonium chloride, 28.7 ml. of 30 per cent aqueous ammonia, and 750 ml. of distilled methanol was allowed to stand for nine days. The methanol was evaporated in vacuo and the residue dissolved in 30 ml. of water saturated with sodium chloride. This solution was extracted with five 50-ml. portions of chloroform and the chloroform extracted with three 100-ml. portions of 1 N hydrochloric acid. The acidic solution was extracted twice with 50-ml. portions of chloroform and then made basic with sodium bicarbonate. The solution was saturated with sodium chloride and extracted with five 100-ml. portions of chloroform. Both chloroform extracts were dried over magnesium sulfate and evaporated to dryness in vacuo. The neutral fraction weighed 1.07 g. and the basic fraction weighed 9.1 g. (78%, crude yield).

A solution of 3 g. of the basic fraction in 150 ml. of 6 N hydrochloric acid was boiled under reflux for 60 hours. The solution was evaporated to about 30 ml. in vacuo and neutralized with dry IRA 45 ( $\text{OH}^-$ ) in absolute ethanol. Ethanol was evaporated, in vacuo, five times from this mixture to remove ammonia. The last ethanol mixture gave a negative test with Nessler's reagent. The amino acid was washed from the resin with water and the solution was passed over an IRA 45 ( $\text{Cl}^-$ ) column. The eluate was evaporated to about 20 ml. in vacuo and the resulting black solution was decolorized with acid-washed charcoal. The suspension was filtered and the filtrate was evaporated to dryness in vacuo to give 2.43 g. of material. Crystallization from water-ethanol afforded 1.85 g. of light gray needles (54%, based on starting ketone). An analytical

sample was prepared by three crystallizations from water-ethanol, m. p. 330-340° dec. (corr.).

Anal.	$C_5H_{11}N_2O_2Cl$	Calc'd:	C, 36.04; H, 6.65; N, 16.81
	(151.60)	Found :	C, 35.71; H, 6.68; N, 17.16

The amino acid had  $R_F$  values of 0.45, 0.52 and 0.24 in BAW, PAW, and PW respectively and gave a purple color with the ninhydrin reagent. It had  $pK_a$  values of 6.5 and 10.1 in water as shown by potentiometric titration. The  $pK_a$  value of the acidic group was not determined (34). A nuclear magnetic resonance spectrum of the amino acid in deuterium oxide (DSS) (c 15%) showed four protons in a group of six peaks near 7.4  $\tau$  and two protons in a group of six peaks near 6.2  $\tau$ .

The benzoyl derivative of 3-aminopyrrolidine-3-carboxylic acid hydrochloride was prepared by a general method for the preparation of N-benzoyl amino acids (17). The amino acid (0.500 g., 3.03 mmole) was dissolved in 2 N sodium hydroxide (3.1 ml.). Alternately there was added to this solution 1.740 g. (12.4 mmole) of benzoyl chloride in five portions and 6.4 ml. of 2 N sodium hydroxide in five portions such that the reaction mixture remained alkaline. The reaction was stirred magnetically throughout the addition and for an additional 30 minutes. The solution was acidified with concentrated hydrochloric acid and extracted with ether. The ether was evaporated to dryness in vacuo and the residue was heated under reflux with 10 ml. of carbon tetrachloride to dissolve benzoic acid. The white residue was crystallized from 50 per cent aqueous-ethanol to give 350 mg. (34%) of white needles, m. p. 222-225° (corr.). The benzoyl derivative was crystallized twice from 50 per cent ethanol

for analysis, m. p. 223-225° (corr.).

<u>Anal.</u> C <sub>19</sub> H <sub>18</sub> N <sub>2</sub> O <sub>4</sub> (338.37)	Calc'd: C, 67.44; H, 5.36; N, 8.28 Found : C, 67.27; H, 5.24; N, 8.18
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The infrared spectrum of the dibenzoyl derivative, in a potassium bromide pellet, showed strong absorbance at 5.80  $\mu$  and 6.08  $\mu$ .

The nuclear magnetic resonance spectrum in deuterium oxide (DSS) (c 10%) with potassium carbonate added to effect solution showed peaks at 2.47  $\tau$  (10 H, singlet), 5.93  $\tau$  (4 H, poorly resolved multiplet) and 7.40  $\tau$  (2 H, poorly resolved multiplet).

A saturated aqueous solution of p-hydroxyazobenzene-p'-sulfonic acid (PHABS) was added to a solution of 100 mg. (0.066 mmole) of 3-aminopyrrolidine-3-carboxylic acid hydrochloride in 0.5 ml. of water. Enough PHABS solution was added to give a deep red solution. The mixture was heated to boiling and enough water was added to give a clear solution. On cooling, yellow plates formed and these were collected, m. p. 256-260° dec. (corr.). An analytical sample was prepared by crystallizing the PHABS derivative two times from water. With each crystallization, 1 ml. of saturated aqueous PHABS solution was added to the hot water solution of the PHABS derivative. Excess PHABS was removed by washing the crystals with 15 1-ml. portions of distilled water.

<u>Anal.</u> C <sub>29</sub> H <sub>30</sub> N <sub>6</sub> O <sub>10</sub> S <sub>2</sub> (686.72)	Calc'd: C, 50.72; H, 4.44; N, 12.24; S, 9.34 Found : C, 50.00; H, 4.42; N, 12.37; S, 9.54
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A solution of 500 mg. (3.87 mmole) of 1-aminocyclopentanecarboxylic acid and 325 mg. (7.75 mmole) of cyanamide in 25 ml. of redistilled



1-butanol was heated under reflux for 48 hours. The solution was cooled and allowed to stand at room temperature. The resulting white crystals were collected, washed with ether, and found to weigh 71 mg. (8%), m. p. 313-315°. The compound was crystallized twice from 1-butanol for analysis, m. p. 320-321° (corr.). Some sublimation was observed starting at 270°. The infrared spectrum of this compound as a nujol mull showed a strong absorbance at 6.01  $\mu$  with a shoulder at 5.83  $\mu$ . The compound gave negative tests with ninhydrin and Weber reagents.

Anal.  $C_{12}H_{18}N_2O_2$   
(222.28)

Calc'd: C, 64.84; H, 8.16; N, 12.61  
Found : C, 64.72; H, 8.13; N, 12.82

Oxidation of Viomycin.-- A solution of 20 mg. (0.093 mmole) of viomycin hydrochloride, 5 ml. of water, and 9.3 ml. of 0.1 M silver permanganate was allowed to stand at room temperature for 24 hours. The manganese dioxide and silver oxide were removed by filtration and five per cent hydrochloric acid was added to the colorless filtrate. The resulting precipitate was removed by filtration, and the filtrate was heated on the steam bath for 30 minutes. The acidic solution was neutralized with IRA 45 (OH<sup>-</sup>) and the eluate from this resin was passed over an IRA 45 (Cl<sup>-</sup>) column. The eluate from this column was evaporated to dryness in vacuo and paper chromatograms were run on the residue. In BAW, the material had  $R_F$  values of 0.66, 0.30 and 0.20 with ninhydrin reagent and 0.66, 0.51 and 0.30 with Weber reagent. In PAW, the material had  $R_F$  values of 0.51 and 0.45 with ninhydrin reagent and 0.57 with Weber reagent. Guanidosuccinic acid had  $R_F$  values of 0.51 and 0.71 in BAW and PAW respectively.

Four reactions were run using 20 mg. (0.093 mmole) of viomycin hydrochloride and 18.6 ml., 9.3 ml., 4.7 ml., and 18.6 ml. of 0.1 M silver permanganate. The first three reactions were carried out at 75° for 40 hours, 6 hours and 3 hours respectively, at which times the solutions were discolored. The last reaction was carried out at 25° for 48 hours and five per cent formic acid was added to decompose excess permanganate. Each of these reactions was treated in the same way as the previously described reaction. Paper chromatograms were run and found to be very complex. No spot which was Weber-positive, ninhydrin-negative and which had the same  $R_F$  value as guanidosuccinic acid was observed in either BAW or PAW.

A solution of 10 mg. of viomycin hydrochloride and 0.5 ml. of 1:1 (v/v) nitric acid was heated on a steam bath for one hour. After evaporating the solution to dryness in vacuo, paper chromatograms were run on the residue in BAW and sprayed with Weber reagent. Guanidine hydrochloride and guanidosuccinic acid were run on the same sheet. No spot of the same  $R_F$  value of guanidosuccinic acid (0.51) was observed for the oxidation mixture but a spot of the same  $R_F$  value (0.66) and color (orange) as guanidine was observed.

A solution of 10 mg. of guanidosuccinic acid in 0.5 ml. of 1:1 (v/v) nitric acid was treated in the manner described and showed only a spot of  $R_F$  0.66 in BAW when sprayed with Weber reagent.

A solution of 10 mg. (0.048 mmole) of viomycin hydrochloride and 5 ml. of one per cent potassium permanganate in ten per cent sulfuric acid was heated to 60° and held at this temperature for ten minutes. Oxalic acid was added to decompose the excess permanganate and the

solution was heated on a steam bath for 30 minutes. The solution was cooled to room temperature; barium carbonate was added to neutralize sulfuric acid and precipitate sulfate and oxalate ions. The mixture was filtered and the filtrate was evaporated to dryness in vacuo. A paper chromatogram was run in BAW with guanidosuccinic acid on the same sheet. The chromatogram was sprayed with Weber and ninhydrin reagents; no spot developed with the ninhydrin reagent. With the Weber reagent,  $R_F$  values of 0.61, 0.51, 0.37 and 0.25 were observed. Guanidosuccinic acid had an  $R_F$  value of 0.51.

To a solution of 500 mg. (2.43 mmole) of viomycin hydrochloride in 12 ml. of water was added 100 ml. (6.3 mmole) of one per cent potassium permanganate in ten per cent sulfuric acid. The solution was heated to 60° and held at this temperature for ten minutes. Oxalic acid was added to reduce the excess permanganate and the solution was heated on a steam bath for 30 minutes. The solution was cooled to room temperature; barium carbonate was added to neutralize the sulfuric acid and precipitate sulfate and oxalate ions. The suspension was heated on the steam bath for five minutes to ensure precipitation and the mixture was filtered. The filtrate was evaporated to dryness in vacuo and the residue weighed 0.67 g.

This material was slurried with 2 ml. of BAW and applied to a cellulose column which was 102 cm. high, 1.8 cm. inside diameter and contained 135 g. of cellulose. Fractions were collected which contained about 2.5 ml. each. These fractions were evaporated at 90° and dried in a desiccator under vacuum overnight. About 0.5 ml. of water was then added to each fraction and chromatograms were run in BAW and sprayed with Weber reagent.

Table 1 summarizes the data obtained. The  $R_F$  values of guanidinosuccinic acid and viomycin hydrochloride were found to be 0.51 and 0.25 respectively in BAW.

Table 1. Oxidation of Viomycin; Cellulose Chromatography

Fraction	Pooled Fraction Number	Weight, grams	Wt. (mg.)/tube	$R_F$ (BAW)	Weber Color
1-6	1	0.010	1.7	Blank	
7-11	2	0.049	9.9	0.61	Pink
12-17	3	0.089	14.8	0.61, 0.55	Pink, yellow
18-21	4	0.053	13.2	0.61, 0.55, 0.51	Pink, yellow, pink
22-29	5	0.083	10.3	0.61, 0.51	Pink, pink
30-38	6	0.075	8.3	0.61, 0.51	Pink, pink
39-42	7	0.019	4.9	0.61, 0.51, 0.37	Pink, pink, brown
43-49	8	0.024	3.4	0.37	Brown
50-60	9	0.028	2.5	0.37, 0.25	Brown, pink
61-78	10	0.037	2.1	0.25	Pink
79-120	11	0.207	5.0	0.25	Pink

Paper chromatograms were run on pooled fractions 5, 6, and 7 in BAW, PAW, and FW and sprayed with Weber reagent. Guanidinosuccinic acid was chromatographed on the same sheets. The  $R_F$  values for fractions 5 and 6 were the same. These values were 0.05, 0.63 and 0.92 (FW), 0.61 and 0.51 (BAW), and 0.70 and 0.79 (PAW). The  $R_F$  values for guanidinosuccinic acid were 0.33, 0.51, and 0.71 in FW, BAW, and PAW respectively.

Fractions 5 and 6 were combined (158 mg.) and dissolved in 10 ml. of saturated aqueous barium hydroxide solution; the solution was heated on a steam bath for 24 hours. The solution was cooled to room temperature and acidified with 1 N sulfuric acid. Barium carbonate was added to neutralize the sulfuric acid and precipitate sulfate ions. The

precipitate was removed by filtration and the filtrate was evaporated to dryness in vacuo. Paper chromatograms were run in BAW, PAW and PW with aspartic acid on the same sheets. The chromatograms were sprayed with ninhydrin reagent. The  $R_F$  values for the hydrolysate were: 0.32 and 0.48 (PW), 0.50 and 0.65 (BAW), and 0.44 (PAW). The  $R_F$  values for aspartic acid were 0.19, 0.28, and 0.77 in PW, BAW, and PAW respectively.

Preparation of Guanidosuccinic Acid.--- The method employed for the preparation of guanidosuccinic acid was a modification of the method of Morgue (26). To a solution of 5.000 g. (0.037 mole) of aspartic acid in 27 ml. (0.4 mole) of concentrated ammonium hydroxide was added 13.600 g. (0.075 mole) of S-ethyl-iso-thiourea hydrobromide (27). The solution was allowed to stand at room temperature for two days.

The pH was then adjusted to 3.3 with concentrated hydrochloric acid. The temperature of the solution was maintained between 15° and 30° during the neutralization. The solution was cooled and the white crystals which formed were collected. They were dissolved in concentrated ammonium hydroxide and the pH was adjusted to 3.3 with concentrated hydrochloric acid, keeping the temperature between 15° and 30°. After cooling the solution, the crystals which formed were collected, washed with cold water, and air dried. There was obtained 2 g. (31%) of material. An analytical sample was prepared by the same procedure but using distilled, constant boiling hydrochloric acid. The white crystals were collected and washed with cold distilled water to remove hydrochloric acid and ammonium chloride. The analytical sample was dried at room temperature and 0.3 mm. for 48 hours. It was a white crystalline solid, m. p. 175° (lit. (26). 175°) if the hot

stage was heated rapidly; when melted in the usual way the analytical sample melted from 175 to 220°. Using the solvent systems BAW, PAW, and PW, and spraying with Weber reagent, one pink spot was observed in each system, of  $R_F$  values 0.51, 0.71, and 0.33 respectively. The analytical sample failed to give a color with ninhydrin reagent.

<u>Anal.</u> $C_5H_9N_3O_4$	Calc'd: C, 34.28; H, 5.18; N, 23.99
(175.14)	Found : C, 34.08; H, 5.21; N, 23.83

In another experiment, the same amounts of reactants as in the previous preparation were mixed and allowed to stand for two days. The pH of the solution was adjusted to 3.3 with concentrated hydrochloric acid. The solution became hot during the neutralization. The crystals which formed on cooling were collected and dissolved in concentrated ammonium hydroxide. The pH of the solution was adjusted to 3.3 as before. The crystals which formed were collected, washed with cold water, and air dried. There was obtained in this way 2.5 g. of material. This material showed two pink spots,  $R_F$  0.0 and 0.51, on paper chromatograms which were run in BAW and sprayed with Weber reagent. The solid was crystallized twice from hot water to give 0.75 g. of material. An analytical sample was prepared by again crystallizing the compound from hot water. The analytical sample was dried at 80° and 1 mm., m. p. 280° (dec.). Paper chromatograms were run in PW, PAW, and BAW and sprayed with the Weber reagent. Only one pink spot of  $R_F$  0.0 was observed in each solvent system. The compound gave a negative ninhydrin test.

<u>Anal.</u> $C_5H_7N_3O_3$	Calc'd: C, 38.22; H, 4.49; N, 26.74
(157.12)	Found : C, 38.41; H, 4.44; N, 26.77

Ozonolysis of Acetylviomycin.-- Ozone generated by a Welsbach Model T-23 ozone generator was bubbled through a solution of 50 mg. (0.24 mmole) of acetylviomycin in 12 ml. of distilled water for one hour at 5-10°. Nitrogen was then bubbled through the solution to remove ozone; the solution was then added to a solution of 3 ml. of 30 per cent hydrogen peroxide and five drops of concentrated sulfuric acid. The resulting solution was heated under reflux for two hours, cooled, and neutralized with IRA 45 (OH<sup>-</sup>). The resin was removed by filtration and the filtrate was passed over on IRA 45 (Cl<sup>-</sup>) column. The eluate was evaporated to dryness in vacuo and paper chromatograms were run in BAW and sprayed with ninhydrin and Weber reagents. Four spots were observed of R<sub>F</sub> 0.0, 0.24, 0.38 and 0.57. All spots gave a positive test with both spray reagents. Acetylviomycin and viomycin were run on the same sheet and had R<sub>F</sub> values of 0.57 and 0.31 respectively. All attempts to crystallize the residue failed.

The residue was dissolved in 5 ml. of aqueous saturated barium hydroxide solution and heated on a steam bath for 48 hours. The reaction mixture was cooled to room temperature and 1 N sulfuric acid was added until the pH of the solution was about 2. The mixture was centrifuged and the supernatant liquid was neutralized with IRA 45 (OH<sup>-</sup>). The resin was removed by filtration and the filtrate was passed over an IRA 45 (Cl<sup>-</sup>) column. The eluate was evaporated to dryness in vacuo to leave 40 mg. of white-brown solid. The solid was dissolved in 0.5 ml. of water and paper chromatograms were run in BAW, PAW, and PW. A sample of  $\alpha,\beta$ -diaminopropionic acid was run on the same sheet in each solvent system. The

chromatograms were sprayed with ninhydrin reagent and the material from acetylviomycin showed  $R_F$  values of 0.64 in PAW, 0.14, 0.25, 0.36, and 0.50 in PW, and 0.38, 0.50, and 0.66 in BAW.  $\alpha$ ,  $\beta$ -Diaminopropionic acid had  $R_F$  values of 0.54 in PAW, 0.10 in PW, and 0.31 in BAW. A pink color developed at room temperature with  $\alpha$ ,  $\beta$ -diaminopropionic acid while no such behavior was observed with the material from acetylviomycin.

Properties of Pyrrole-2-carboxylic Acid.-- Commercially available pyrrole-2-carboxylic acid was crystallized from benzene to give white plates, m. p. 187-189° dec. (corr.) (lit. (28) 191-192°). The melting behavior was dependent on the rate of heating and if the hot stage was allowed to cool after decomposition had started, decomposition would continue even at 160°. Pyrrole-2-carboxylic acid gave an intense purple color with the Ehrlich reagent.

Preparation and Properties of Pyrrole-3-carboxylic Acid.-- Pyrrole-3-carboxylic acid was prepared by a modification of the method of Rinkes (29). Aminoacetaldehyde hydrochloride was prepared by the method of Fischer (30). The nuclear magnetic resonance spectrum of the aminoacetal, as the neat liquid (TMS), showed peaks at 5.63  $\tau$  (1 H, triplet,  $J = 5.5$  cps.), 6.45  $\tau$  (4 H, two almost superimposed quartets,  $J = 3.7$  cps.), 7.37  $\tau$  (2 H, doublet,  $J = 5.5$  cps.) and 8.87  $\tau$  (6 H, triplet,  $J = 8.0$  cps. and 2 H, singlet). To a solution of 50 g. (0.38 mole) of aminoacetal in 18 ml. of water was added 300 ml. of concentrated hydrochloric acid at 0-5°. The resulting red solution was allowed to stand at room temperature for four hours; water and hydrochloric acid were removed by evaporation in vacuo at 35°. The nuclear magnetic resonance spectrum of the resulting red oil



was determined in deuterium oxide solution (DSS) and showed peaks at  $4.58 \tau$  (1 H, triplet,  $J = 5.5$  cps.) and  $6.87 \tau$  (2 H, doublet,  $J = 5.5$  cps.).

To a solution of 10.5 g. (0.05 mole) of the sodium salt of diethyl oxalylacetate and 6.7 g. (0.12 mole) of potassium hydroxide in 60 ml. of water was added a solution of 9 g. (0.1 mole) of aminoacetaldehyde hydrochloride in 50 ml. of water. The addition was carried out at  $0-10^{\circ}$ . The reaction mixture was allowed to stand at room temperature for six hours and then cooled and acidified with 6 N hydrochloric acid. The acidic solution was extracted five times with chloroform, the chloroform was dried over magnesium sulfate, and the chloroform was evaporated in vacuo. The resulting brown solid was crystallized from 50 per cent aqueous ethanol to give 1.5 g. (18%) of 3-carbethoxypyrrole-2-carboxylic acid, m. p.  $148-149^{\circ}$  subl. (corr.) (lit. (29)  $146-147^{\circ}$ ). The nuclear magnetic resonance spectrum of the ester-acid was determined in deuterium oxide solution (DSS), with anhydrous potassium carbonate added to effect solution. It showed peaks at  $3.08 \tau$  (1 H, doublet,  $J = 2.8$  cps.),  $3.38 \tau$  (1 H, doublet,  $J = 2.8$  cps.),  $5.68 \tau$  (2 H, quartet,  $J = 7.3$  cps.), and  $8.67 \tau$  (3 H, triplet,  $J = 7.0$  cps.).

A mixture of 1.1 g. (6.5 mmole) of 3-carbethoxypyrrole-2-carboxylic acid, 5 ml. of freshly distilled quinoline, and 500 mg. of copper chromite catalyst was heated in an oil bath. The copper chromite catalyst was prepared by the method of Lazier (31). A gas was evolved at  $160^{\circ}$  and the temperature was slowly raised to  $200^{\circ}$ . The temperature was maintained at  $200^{\circ}$  until no more gas was evolved. The mixture was allowed to cool to room temperature and 20 ml. of ether was added. The catalyst was removed by filtration and the filtrate was washed with 1 N hydrochloric acid to

removed quinoline. The ether solution was washed with water, dried over magnesium sulfate, and evaporated to dryness in vacuo. The brown oil which remained weighed 540 mg. A nuclear magnetic resonance spectrum was determined in carbon tetrachloride solution (TMS) and showed peaks at 2.59  $\tau$  (1 H, poorly resolved multiplet), 3.31  $\tau$  (1 H, poorly resolved multiplet), 3.41  $\tau$  (1 H, poorly resolved multiplet), 5.75  $\tau$  (2 H, quartet,  $J = 7.0$  cps.), and 8.72  $\tau$  (3 H, triplet,  $J = 7.0$  cps.).

A solution of 460 mg. of crude ethyl pyrrole-3-carboxylate in 3 ml. of ethanol was heated on a steam bath for 2.5 hours with a solution of 2 g. of potassium hydroxide in 5 ml. of water. The solution was evaporated to dryness in vacuo. Concentrated hydrochloric acid was added and the mixture was extracted six times with anhydrous ether. The ether was dried over magnesium sulfate and evaporated to dryness in vacuo. The residue was crystallized from benzene to give 220 mg. (33% from 3-carbethoxypyrrole-2-carboxylic acid) of white needles, m. p. 146-147° corr. (lit. (29) 147-148°). Pyrrole-2-carboxylic acid gave an intense purple color with the Ehrlich reagent.

Barium Hydroxide Hydrolysis of Viomycin.-- A solution of 100 mg. (0.49 mmole) of viomycin hydrochloride in 10.7 ml. of saturated aqueous barium hydroxide solution was heated on a steam bath for 77 hours. Sulfuric acid-washed nitrogen was passed over the solution; evolved bases were trapped by allowing the effluent nitrogen to bubble through about 100 ml. of 1 N hydrochloric acid during the reaction. The acidic solution was evaporated to dryness in vacuo and the residue was dissolved in 0.5 ml. of water. The nuclear magnetic resonance spectrum of

this solution (TMS, external standard) showed no peaks above 5  $\tau$ . Two sharp peaks of equal intensity were observed at 1.95  $\tau$  and 2.75  $\tau$ .

The alkaline reaction mixture was cooled to room temperature and centrifuged. The clear solution was passed over an IRC 50 ( $H^+$ ) column, and the eluate was evaporated to dryness in vacuo. Approximately 100 mg. of tan solid was obtained. This solid was stirred with hot absolute ethanol and the mixture was centrifuged. The ethanol solution was evaporated to dryness in vacuo; the resulting solid was heated under reflux with 8 ml. of dry benzene for one hour. The benzene suspension was centrifuged and the benzene was concentrated to about 2 ml. The solution was cooled and the resulting white crystals were collected and air-dried, 12 mg., m. p. 170-175° dec.). The melting behavior was dependent on the rate of heating and if the hot stage was allowed to cool after decomposition had started decomposition would continue even at 160°. This compound was found to give an intense purple color with the Ehrlich reagent.

The infrared spectra of pyrrole-2-carboxylic acid, pyrrole-3-carboxylic acid, and the compound from viomycin were taken using potassium bromide pellets. The ultraviolet spectra of the unknown compound, pyrrole-2-carboxylic acid, and pyrrole-3-carboxylic acid were recorded in 95 per cent ethanol solution. The respective absorptions were:  $\lambda_{max}$ . 260  $m\mu$ ,  $\epsilon$ , 10,000;  $\lambda_{max}$ . 261  $m\mu$ ,  $\epsilon$ , 11,000; and  $\lambda_{max}$ . 245  $m\mu$ ,  $\epsilon$ , 4,300, respectively. The nuclear magnetic resonance spectra were recorded in deuterium oxide solutions ( $\leq 1.5\%$ ) (TMS, external standard). The unknown compound and pyrrole-2-carboxylic acid showed peaks at 2.98  $\tau$  (1 H, multiplet), 3.18  $\tau$  (1 H, multiplet), and 3.68  $\tau$  (1 H, multiplet).

Pyrrole-3-carboxylic acid showed peaks at 2.38  $\tau$  (1 H, multiplet), 3.05  $\tau$  (1 H, multiplet), and 3.33  $\tau$  (1H, multiplet).

A paper chromatogram of the unknown compound was run in a solvent system composed of 1-butanol, acetic acid, and water in a ratio of 5:4:1 (v/v) respectively. Pyrrole-2-carboxylic acid and pyrrole-3-carboxylic acid were run on the same sheet. The chromatograms were sprayed with Ehrlich's reagent. The unknown and pyrrole-2-carboxylic acid showed one brown spot,  $R_F$  value of 0.75. Pyrrole-3-carboxylic acid showed one purple spot with an  $R_F$  value of 0.71.

Sodium Hydroxide Hydrolysis of Viomycin.-- In a sublimation apparatus was placed 100 mg. (0.49 mmole) of viomycin hydrochloride, 3.7 g. of sodium hydroxide pellets and seven drops of water. The apparatus was placed in a sand bath at 80° and the temperature was slowly raised to 100°. When no more sublimate appeared, heating was stopped. The time required was about one hour. The sublimate was dissolved in about 20 ml. of 0.1 N hydrochloric acid and the ultraviolet spectrum was recorded. About 1 mg. of authentic 2-aminopyrimidine was dissolved in 20 ml. of 0.1 N hydrochloric acid and the ultraviolet spectrum was recorded. Both spectra showed maximum absorptions at 301 m $\mu$  and 226 m $\mu$ . The shape and intensity of the two peaks was the same for each spectrum. The literature values for 2-aminopyrimidine are 301 m $\mu$ ,  $\epsilon$ , 4,000, and 221 m $\mu$ ,  $\epsilon$ , 15,000, in 0.1 N hydrochloric acid (32).

The alkaline reaction mixture was cooled to room temperature and dissolved in 30 ml. of water. The solution was passed over an IRC 50 (H<sup>+</sup>) column and the eluate was evaporated to dryness in vacuo. About 145 mg.

of tan solid was obtained. The solid was heated under reflux with 10 ml. of dry benzene for 30 minutes. The hot suspension was centrifuged and the benzene solution was evaporated to dryness. The benzene-soluble fraction was dissolved in 50 ml. of 95 per cent ethanol and the ultraviolet spectrum was recorded. The compound exhibited a peak at 261  $m\mu$  with an absorbance of 1.3.

The benzene-insoluble material was dissolved in 2 ml. of water and paper chromatograms were run on the solution; glycine was run on the same sheet. There was one ninhydrin-positive spot with the same  $R_F$  value as glycine in each of four solvent systems. The solvent systems used were: BAW, 77 per cent ethanol-water; *t*-amyl alcohol, *n*-propyl alcohol, 4:1:1 (v/v); and a phosphate buffer composed of a 1.5 *M* solution of sodium dihydrogen phosphate with enough 1 *N* hydrochloric acid added to make the solution pH 6. The  $R_F$  values for each solvent system were 0.52, 0.27, 0.11 and 0.24 respectively. The nuclear magnetic resonance spectrum of the benzene-insoluble material was recorded in water solution (TMS external standard) and showed peaks at 6.21  $\tau$  and 6.13  $\tau$ . Glycine in water solution (TMS external standard) showed a single peak at 6.35  $\tau$ .

To a solution of the benzene-insoluble material in 2 ml. of water containing 125 mg. of sodium bicarbonate was added a solution of 125 mg. of 2,4-dinitrofluorobenzene in 4 ml. of absolute ethanol. The reaction mixture was stirred magnetically for two hours at room temperature and placed in the refrigerator for 12 hours. The ethanol was evaporated in vacuo and 1 *N* hydrochloric acid was added to the residue. The aqueous suspension was extracted with two 20 ml. portions of benzene, which predominately removed 2,4-dinitrophenol and excess 2,4-dinitrofluorobenzene.

The aqueous solution was then extracted with ethyl acetate until the ethyl acetate layer was no longer yellow. The ethyl acetate was dried over magnesium sulfate and evaporated to dryness in vacuo. The residue was crystallized from water and gave 1 mg. of yellow needles, m. p. 194-198° dec. (corr.). Authentic 2,4-dinitrophenylglycine was prepared and crystallized in the same way, m. p. 195-200° dec. (corr.). (Lit. (17) 200-201°). Paper chromatograms were run on the 2,4-dinitrophenyl derivative of the material from viomycin and 2,4-dinitrophenylglycine. The same  $R_F$  value (yellow spot) was observed in BAW, 77% ethanol-water and the phosphate buffer. The  $R_F$  values for each solvent system were 0.50, 0.48 and 0.55 respectively. The infrared spectrum of 2,4-dinitrophenylglycine and the 2,4-dinitrophenyl derivative of the material from viomycin, in potassium pellets, was recorded.

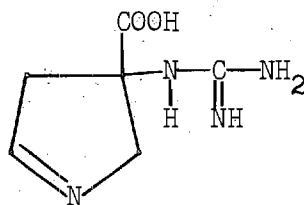
Acetyl-dl-Proline.-- Acetyl-dl-proline was prepared, as previously recorded (17). In trifluoroacetic acid solution (TMS) (c 25%), the nuclear magnetic resonance spectrum of acetyl-dl-proline showed peaks at 4.92  $\tau$  (1 H, triplet,  $J = 6.0$  cps.), 5.03  $\tau$  (2 H, triplet,  $J = 6.5$  cps.), 7.40  $\tau$  (3 H, singlet) and 7.52  $\tau$  (4 H, poorly resolved multiplet).

Acetylpyrrolidine.-- At 15°, 75 g. (0.75 mole) of acetic anhydride was added dropwise to 35 g. (0.50 mole) of pyrrolidine. After the addition the solution was heated on a steam bath for 30 minutes. Five per cent sodium bicarbonate solution was added to decompose the excess acetic anhydride and neutralize acetic acid; the mixture was extracted with chloroform. The chloroform solution was washed with five per cent hydrochloric

acid, dried over magnesium sulfate and evaporated in vacuo. The residue was distilled, giving 20 g., b. p. 120-122°/30 mm (lit. (33) 108°/15 mm),  $n_D^{20} = 1.4780$  (lit. (33) 1.4778). The infrared spectrum of acetylpyrrolidine showed a strong peak at 5.95  $\mu$ . The nuclear magnetic resonance spectrum of acetylpyrrolidine, as the neat liquid (TMS), showed peaks at 6.64  $\tau$  (4 H, multiplet,  $J = 3.5$  cps.), 8.08  $\tau$  (3 H, singlet) and 8.13  $\tau$  (4 H, triplet,  $J = 3.5$  cps.).

## DISCUSSION

Functional Groups in Viomycidine.\*\* Structure I was proposed for viomycidine early in this research. Many of the experiments undertaken were designed for the purpose of gaining evidence for or against this structure and these experiments will be discussed in terms of structure I.



I

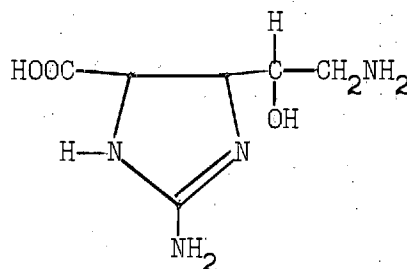
Viomycidine hydrochloride was found to have the formula  $C_6H_{10}N_4O_2 \cdot HCl$  by elemental analysis. The absence of  $O-CH_3$ ,  $N-CH_3$ ,  $C-CH_3$  and primary amino groups was indicated by analysis (6). From the formula it is seen that viomycidine has four rings and/or double bonds. The optical rotation of viomycidine varies with the pH of the solution, being  $-21^\circ$  in acid solution,  $-83^\circ$  in water and  $-135^\circ$  in basic solution (6). Viomycidine has  $pK_a^*$  values of 2.8, 5.87 and 13.4 in 66 per cent dimethylformamide and 5.50 and 12.6 in water (6). It was not possible to determine the  $pK_a$  value of the most strongly acidic function in water by the method used. The  $pK_a$  value of an acidic group is less in water than in 66 per cent dimethylformamide. For example, the  $pK_a$  values in water and

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\* See footnote on page one.



dimethylformamide respectively for the following amino acids are: lysine, 2.18 and 3.70 (34); glutamic acid, 2.10 and 4.00 (34); alanine, 2.45 and 4.30 (34); and streptolidine (II), 2.50 and 3.95 (35).



II

From these data, the lowest  $pK_a$  value for viomycinidine in water is estimated to be about 1.3. The  $pK_a$  values for the carboxylic acid group of normal  $\alpha$ -amino acids in water are  $2.3 \pm .15$  (17). The  $pK_a$  values in water for the carboxylic acid group of ornithine ( $\alpha, \gamma$ -diaminovaleric acid) and  $\alpha, \beta$ -diaminopropionic acid, two amino acids which contain two basic centers, are 1.94 and 1.39 respectively (17). In these amino acids, the basic centers are not too distant from the carboxylic acid group. When the second basic center is further from the carboxylic acid group, as in arginine and lysine ( $pK_a$  value of 2.18 in water for each), the acid is of about the same strength as for normal  $\alpha$ -amino acids. It is very unlikely that there could be any group in viomycinidine with the observed  $pK_a$  value except a carboxylic acid group.

The  $pK_a$  value of 12.6 in water and 13.4 in 66 per cent dimethylformamide indicates the presence of a strongly basic functional group in viomycinidine. In water the guanidine group of arginine has a  $pK_a$  value of 13.2 (17) and the guanidine group of streptolidine (II) has a  $pK_a$

value of 11.30 (35). In 66 per cent dimethylformamide the guanidine groups of  $\alpha$ - and  $\beta$ -guanidinopropionic acid have  $pK_a$  values of 14.7 and 14.5 respectively (34). In 66 per cent dimethylformamide the guanidine group of streptolidine (II) has a  $pK_a$  value of 12.65 (35). Of fifty-six amino acids, none has a  $pK_a$  value greater than 10.7 unless the amino acid contains a guanidine group (17). Therefore, the observed  $pK_a$  value of 12.6 strongly indicates a guanidine group in viomycin.

The pink color observed with viomycin and the Sakaguchi reagent indicates the presence of a mono-substituted guanidine group since the Sakaguchi reagent is specific for mono-substituted guanidines (9,17). Methylguanidine, arginine, guanidinoacetic acid,  $\alpha$ -guanidinopropionic acid,  $\beta$ -guanidinopropionic acid,  $\alpha$ -guanidinobutyric acid and  $\beta$ -guanidinobutyric acid each gave a pink color with the Sakaguchi reagent. This color was stable for at least thirty minutes. Creatine, creatinine, 1,2,3-triphenylguanidine and guanidine failed to give a color with the reagent and 1,3-diphenylguanidine gave a gray color with the reagent. The Weber reagent for guanidine is not as specific as the Sakaguchi reagent, giving an orange-pink color with acetamidine and S-ethyl-iso-thiourea as well as giving a pink color with substituted guanidines; guanidine gives an orange color with this reagent. However, a pink color as given by viomycin, usually indicates a mono- or N,N'-di-substituted guanidine. Tri-substituted and N,N-di-substituted guanidines usually give no color or a blue color with the Weber reagent (9, 36).

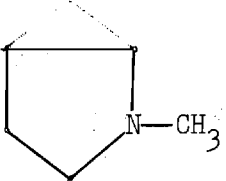
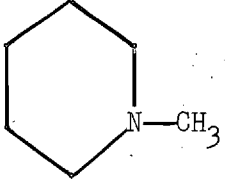
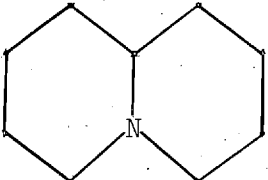
With the assignment of carboxylic acid and mono-substituted guanidine groups to viomycin there remains a  $C_4H_5N$  moiety to account for. Two sites of unsaturation have been assigned, leaving two rings and/or

double bonds. Although no pure dihydroviomycidine could be isolated, viomycidine did absorb about one mole of hydrogen per mole of viomycidine. This indicates the presence of one double bond and one ring in viomycidine.

It has been found that the ultraviolet absorbance of the free base of a tertiary amine is considerably greater than the absorbance of the conjugate acid. (7, 34). With primary and secondary amines this difference in absorbance of the two species is not nearly as pronounced. This furnishes a convenient method for determining if a given basic group, within a molecule containing other ionizable groups, is a tertiary amine or not. The method consists of determining the ultraviolet spectrum of the amine in a solution whose pH is about two pH units higher than the pKa value for the amine. The ultraviolet spectrum of the amine is then determined in a solution whose pH is about two pH units lower than the pKa value for the amine. The spectrum of the solution of lower pH is then subtracted from the spectrum of the solution of higher pH, effectively giving the difference in absorbance of the basic and acidic forms of the amine. With some tertiary amines a maximum in the absorbance is observed at about 210 mμ when the determination is done in this way. With other tertiary amines no maximum is observed but rather just a large absorbance. Table 2 summarizes some data obtained in this way.

Viomycidine exhibits  $\lambda_{\text{max}}$  of 210 mμ,  $\epsilon$ , 2,400 when the ultraviolet spectrum of the acidic form of the group with a pKa value of 5.50 is subtracted from the ultraviolet spectrum of the basic form of this group.

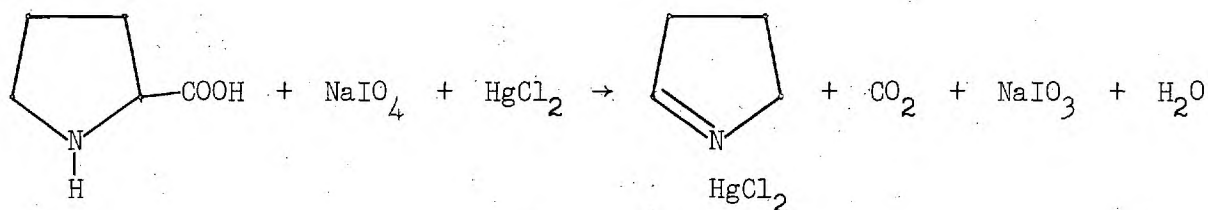
Table 2. Differential Ultraviolet Spectra of Some Tertiary Amines.

Compound	$\lambda_{\text{max.}}$	$\epsilon$	Ref.
	214 $m\mu$	2300	(34)
	213 $m\mu$	1600	(34)
	215 $m\mu$	3100	(34)
$(\text{NC-CH}_2\text{-CH}_2)_3\text{N}$ . . . . .	2000 (at 210 $m\mu$ )		(7)
$(\text{NC-CH}_2\text{-CH}_2)_2\text{N-CH}_2\text{CH}_3$ . . . . .	793 (at 210 $m\mu$ )		(7)
$\text{NC-CH}_2\text{-CH}_2\text{-N-(CH}_2\text{-CH}_2)_2$ . . . . .	718 (at 210 $m\mu$ )		(7)
$(\text{NC-CH}_2)_2\text{N-H}$ . . . . .	133 (at 210 $m\mu$ )		(7)
$(\text{CH}_3\text{CH}_2)_2\text{N-H}$ . . . . .	134 (at 210 $m\mu$ )		(7)
$\text{CH}_3\text{CH}_2\text{-NH}_2$ . . . . .	101 (at 210 $m\mu$ )		(7)

The ultraviolet spectrum of triethylamine minus the ultraviolet spectrum of the conjugate acid of this amine revealed  $\epsilon$ , 1,200 at 210  $m\mu$ . Di-n-propylamine had  $\epsilon$ , 400 at 210  $m\mu$  and n-propylamine had  $\epsilon$ , 80 at 210  $m\mu$ . These data and the data given in Table 2 indicate that the weakly basic group ( $pK_a = 5.50$ ) of viomycinidene appears to be a tertiary amine.

It is considered that the small-membered rings (three or four) containing nitrogen are unlikely because of the stability of viomycinidene to acid. Thus it was considered likely that the remaining  $C_4H_5N$  part of viomycinidene (aside from the mono-substituted guanidine and carboxylic acid functions) was present as a five-membered ring containing a nitrogen atom and a double bond. Considering this unit as a possibility, the only conceivable way that the amine function can be tertiary is for it to be present as an imine; the unit would then be a  $\Delta^1$ -pyrroline.

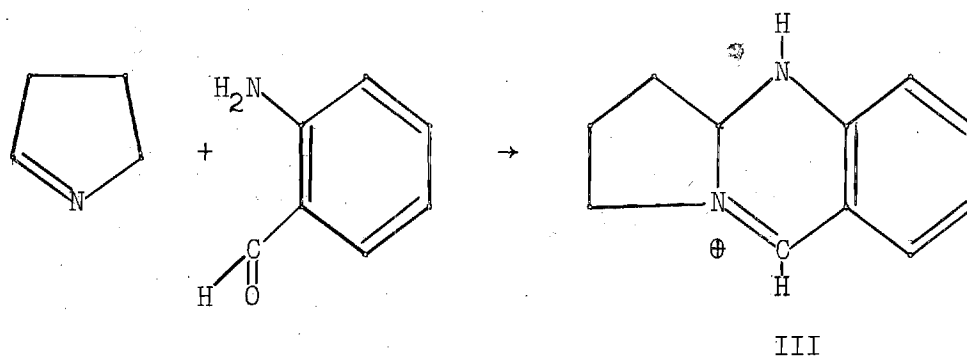
The mercuric chloride complex of  $\Delta^1$ -pyrroline was prepared to furnish a model compound. The method used was the periodate oxidation of proline in the presence of mercuric chloride (20); the equation for the reaction is



The mercuric chloride complex of  $\Delta^1$ -pyrroline was a white solid which was insoluble in water and organic solvents. It showed a strong band at 6.12  $\mu$  in the infrared region indicative of a carbon-nitrogen double bond and did not show a band in the 2.5  $\mu$ -3.3  $\mu$  region, indicating the absence of a nitrogen-hydrogen bond.

The stability of  $\Delta'$ -pyrrolines have been previously investigated (23, 37, 38). One of the methods of synthesis of  $\Delta'$ -pyrrolines involves heating a  $\gamma$ -aminoaldehyde or ketone with 6 N hydrochloric acid for several hours.  $\Delta'$ -Pyrroline itself has been shown to be stable at 100° in a solution of pH 1 for at least eight minutes. No loss of  $\Delta'$ -pyrroline was observed during this time so  $\Delta'$ -pyrroline would probably be stable for longer periods of time, although this was not reported (23). In this work it has been found that  $\Delta'$ -pyrroline in trifluoroacetic acid solution undergoes no change during at least one week. From this discussion it is seen that the stability of  $\Delta'$ -pyrrolines toward acid would not exclude the presence of a  $\Delta'$ -pyrroline moiety in viomycin.

The reaction of *o*-aminobenzaldehyde with  $\Delta'$ -pyrrolines to form a yellow compound has been previously described (23, 37, 38, 39). The general reaction to give a compound of type III can be formulated as follows:



As a qualitative color test, it was found that the following known  $\Delta'$ -pyrrolines gave a yellow color with *o*-aminobenzaldehyde: the mercuric chloride complex of  $\Delta'$ -pyrroline (20), 2,3-dimethyl- $\Delta'$ -pyrroline-5-carboxylic acid hydrochloride (21), 2-ethyl-3,3-diphenyl- $\Delta'$ -pyrroline

(24), 2-methyl-3,3-diphenyl- $\Delta^1$ -pyrroline (24), 4,4,5-triphenyl- $\Delta^1$ -pyrroline (24), and 4,4-diphenyl-5-(p-tolyl)- $\Delta^1$ -pyrroline (24).

iso-Butylidineethylamine also gave a yellow color with the reagent but the color faded in about two hours. The color obtained with the  $\Delta^1$ -pyrrolines was stable for at least 24 hours. Viomycin gave a yellow color with the o-aminobenzaldehyde reagent and this color was stable for at least 24 hours.

The ultraviolet and visible spectra of the compound formed from  $\Delta^1$ -pyrroline-5-carboxylic acid and o-aminobenzaldehyde have been previously recorded as having  $\lambda_{\text{max}}$  at 430  $\mu$ , 294  $\mu$ , and 232  $\mu$  (38); in Strecker's work the yellow compound was isolated. The ultraviolet and visible spectra of the yellow color resulting from some known  $\Delta^1$ -pyrrolines and viomycin with o-aminobenzaldehyde were examined in this work. These experiments were semi-quantitative since, while the volume of o-aminobenzaldehyde reagent was known, the exact composition of this reagent was not known. Thus, although the weight of the compound being examined was known, the amount of yellow compound formed was not known. In each of these experiments, an amount of o-aminobenzaldehyde remained and interfered with the spectra; o-aminobenzaldehyde had  $\lambda_{\text{max}}$  at 350  $\mu$  and 260  $\mu$ . 2-Methyl- $\Delta^1$ -pyrroline-5-carboxylic acid and 2,3-dimethyl- $\Delta^1$ -pyrroline-5-carboxylic acid with the o-aminobenzaldehyde reagent showed a shoulder at 420-450  $\mu$ , and  $\lambda_{\text{max}}$  at 296  $\mu$ .  $\Delta^1$ -Pyrroline with the reagent showed a shoulder at 420-450  $\mu$  and a  $\lambda_{\text{max}}$  at 288  $\mu$ . Viomycin with the reagent showed a shoulder at 420-450  $\mu$  and a  $\lambda_{\text{max}}$  at 301  $\mu$ . No peak near 230  $\mu$  was observable, probably because of the excess o-aminobenzaldehyde present. These data are summarized in Figure 6 and indicate the presence of a

$\Delta'$ -pyrroline moiety in viomycidine.

The  $pK_a$  value of 5.5 in water indicates the presence of a weakly basic group in viomycidine. Normal aliphatic amines have  $pK_a$  values of around 10 (17). Some values for known  $\Delta'$ -pyrrolines are 3  $\beta$ ,27-dihydroxy-16  $\beta$ ,22-imino-5,22-(n)-cholestadiene, 5.45 (40); 2-methyl- $\Delta'$ -pyrroline, 7.77 (41); 2-ethyl-3,3-diphenyl- $\Delta'$ -pyrroline, 4.7 (33 per cent dimethylformamide (34));  $\Delta'$ -pyrroline, 5.6 (water) (34); and 2-methyl- $\Delta'$ -pyrroline-5-carboxylic acid, 7.37 (66 per cent dimethylformamide) (34). Therefore the weakly basic character of the remaining nitrogen in viomycidine is consistent with the assignment of a  $\Delta'$ -pyrroline in viomycidine.

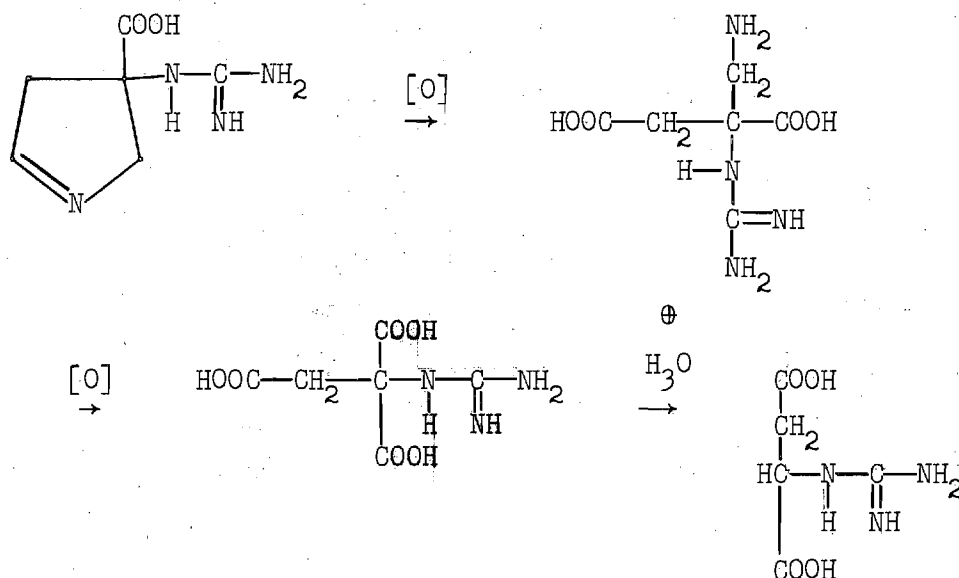
A substituted imine is not a typical tertiary amine analogous to the tertiary amines in table 2. However, a substituted imine is like a tertiary amine in that it does not have a proton covalently bonded to it. Therefore the observed differential ultraviolet spectrum of the weakly basic nitrogen in viomycidine is probably consistent with it being a substituted imine.

In summary, the presence of a  $\Delta'$ -pyrroline moiety in viomycidine is strongly indicated from the  $o$ -aminobenzaldehyde data, the  $pK_a$  data and the data indicating that the weakly basic nitrogen is a tertiary amine.

Attempted Degradation of Viomycidine to Known Compounds.--- Several reaction sequences were sought for the transformation of viomycidine into known compounds. Reactions tried were oxidation, reduction, base hydrolysis and ozonolysis of a derivative of viomycidine. Acidic degradations were not thought feasible since viomycidine had been shown to be fairly stable to acidic conditions (6).



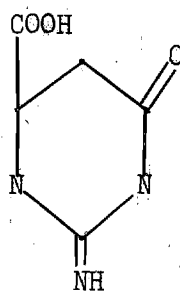
The first reaction attempted was the oxidation of viomycinidine followed by decarboxylation of the proposed intermediate product. The proposed sequence of reactions was as follows:



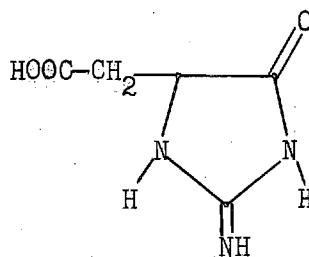
This sequence of reactions was attractive because the synthesis of the final product, guanidinosuccinic acid, had been previously reported (19). Further, guanidinosuccinic acid could be degraded to aspartic acid by alkaline hydrolysis thus facilitating identification of guanidinosuccinic acid. Alkaline hydrolysis of guanidines to give amines is a general reaction of guanidines.

The reported synthesis of guanidinosuccinic acid employed one of the standard methods for the preparation of guanidines and involved the reaction of aspartic acid with S-ethyl-iso-thiourea hydrobromide in concentrated ammonium hydroxide solution. In the preparation of guanidinosuccinic acid by this method, it was found that repeated crystallization of crude guanidinosuccinic acid from hot water gave a compound, m. p.  $280^\circ$ .

This compound gave a positive Weber reaction and had an  $R_F$  value of zero in PAW, PW, and BAW. Elemental analysis indicated that the compound had the formula  $C_5H_7N_3O_3$ , corresponding to the formula of guanidinosuccinic acid minus a mole of water. In the infrared region the compound showed a strong peak at  $5.92 \mu$ . This compound clearly contained a carboxylic acid function because it was insoluble in water but soluble in dilute aqueous ammonia. Thus the compound probably has either structure IV or V. Structure V is considered more likely since, in the formation of lactams, the five-membered ring is generally formed more readily than the six-membered ring.



IV



V

Repeated crystallization of the crude product by solution in concentrated ammonium hydroxide and acidification to pH 3.3 with 6 N hydrochloric acid at room temperature gave a compound with satisfactory analytical data for guanidinosuccinic acid. The compound had  $R_F$  values of 0.51, 0.71, and 0.33 in BAW, PAW, and PW respectively, the paper chromatograms being sprayed with Weber reagent. The literature value for the melting point,  $175^\circ$ , was reproducible if guanidinosuccinic acid was heated rapidly but with slow heating guanidinosuccinic acid melted over a range of  $175-220^\circ$ . Probably dehydration occurred to produce either IV or V.

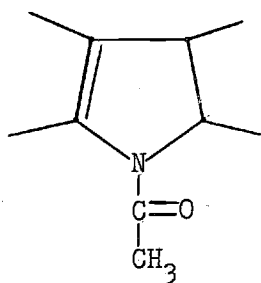
The first attempts to oxidize viomycin were with silver permanganate in water. The oxidant and the essentially neutral media were chosen because of the ease of removal of inorganic ions. Manganese ion would be present as insoluble manganese dioxide and silver ion could be removed by precipitation with hydrochloric acid. After oxidation, the solution was heated in acid to effect decarboxylation. A number of individual reactions were run, using different amounts of oxidant, different temperatures and different reaction times; none produced guanidinosuccinic acid as shown by paper chromatography.

Oxidation of both viomycin and guanidinosuccinic acid with hot nitric acid gave a compound with the same  $R_F$  value as guanidine in BAW. The compound gave the same orange color as guanidine when the paper chromatogram was sprayed with Weber reagent. This was the only compound produced that gave a positive Weber test and no compound was produced which gave a positive ninhydrin test. It was concluded from these data that nitric acid could not be used to oxidize viomycin to guanidinosuccinic acid because guanidinosuccinic acid was oxidized by this reagent.

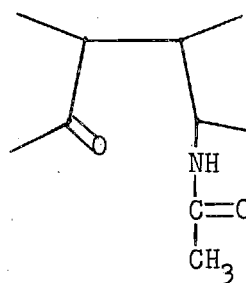
Viomycin was oxidized by potassium permanganate in hot acidic solution; heating of the acidic solution was continued after destroying the excess permanganate. This resulted in the formation of a compound, among others, with the same  $R_F$  value as guanidinosuccinic acid in BAW (Weber reagent). An attempt was made to separate this compound from the other reaction products by cellulose chromatography. None of the fractions from this column contained a compound with the same  $R_F$  value as guanidinosuccinic acid in the PW solvent system. Two of the fractions

contained a compound with the same  $R_F$  value as guanidinosuccinic acid in PAW and BAW. These fractions were hydrolyzed in hot aqueous barium hydroxide solution. No material with the same  $R_F$  value as aspartic acid was observed on paper chromatograms, further proving that no guanidinosuccinic acid had been present in the oxidation products. Since the proposed sequence of reactions appeared reasonable, the absence of guanidinosuccinic acid is an indication that structure I might not be correct for viomycin.

It has been indicated previously that the presence of a  $\Delta^1$ -pyrroline ring in viomycin appeared likely. Uhle and Sallman (40) reported the acetylation of a steroid containing a  $\Delta^1$ -pyrroline moiety to give two compounds, VI and VII, one being an N-acetyl- $\Delta^2$ -pyrroline and the other being a ring-opened N-acetyl- $\gamma$ -aminoketone.

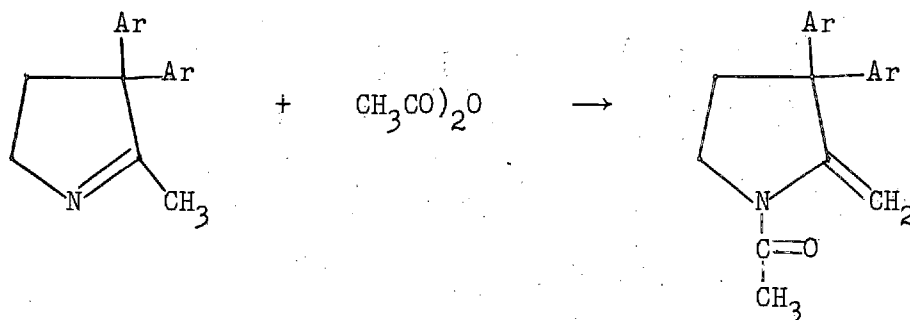


VI

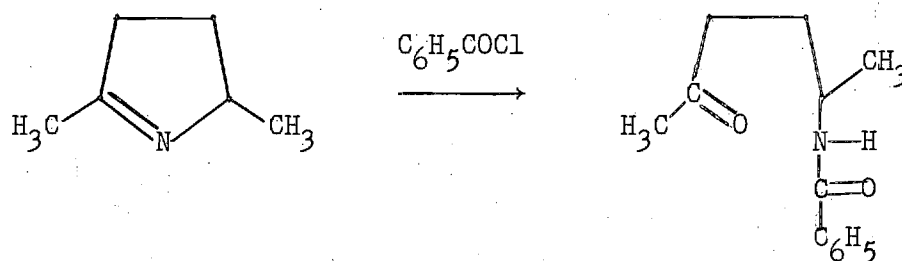


VII

Janssen and co-workers reported the same type of reactions;  $\Delta^2$ -pyrrolines were not obtained since the 3-position was blocked by two aryl substituents. Rather, the double bond was shifted to an exocyclic position as seen in the following reaction:

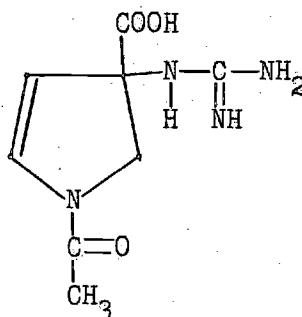


The same type of product was obtained if a benzoyl group was introduced rather than an acetyl group. No reaction was observed with 2,3,3-triphenyl- $\Delta'$ -pyrrolidine where the double bond cannot shift to an exocyclic position. No ring-opened compounds were observed with any of these  $\Delta'$ -pyrrolidines (42). Evans reported the following reaction to give a ring-opened compound (43).



Since there seemed to be adequate precedence for this type of reaction in the literature, it was thought that an acetyl derivative of viomycin might be obtained. An acetyl derivative of viomycin was prepared by the reaction of viomycin hydrochloride with acetic anhydride in cold aqueous ethanol. Under these conditions, guanidines are not acetylated (17). Elemental analysis revealed that acetyl-viomycin

has the formula anticipated,  $C_8H_{12}N_4O_3$ . This indicates the  $\Delta^2$ -pyrroline type structure rather than the ring-opened structure, which would require an additional mole of water in the formula. Acetylviomycidine gave a negative test with ninhydrin reagent, a positive test with Weber reagent and gave only viomycidine when heated with concentrated hydrochloric acid as shown by paper chromatography. These data, the pKa values of 4.86 and 13.0, and the infrared spectra (Figure 3) are consistent with structure VIII for acetylviomycidine. The formation of an N-acetyl derivative of viomycidine further supports the previous conclusion that the weakly basic nitrogen is not bonded to three carbon atoms.

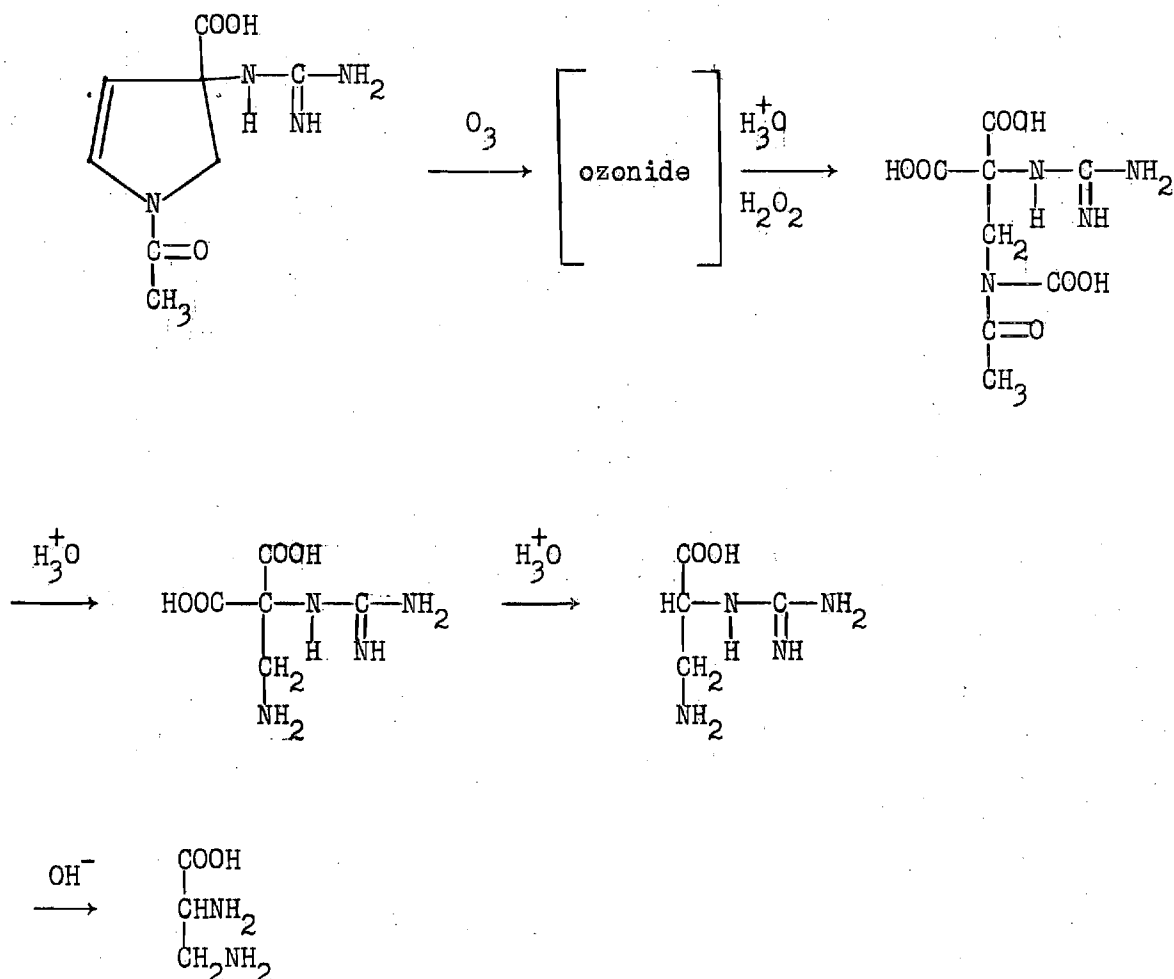


VIII

An attempt was made to prepare the N-acetyl derivative of  $\Delta^1$ -pyrroline by the reaction of acetic anhydride with a solution of  $\Delta^1$ -pyrroline of pH 3.5. While no pure compound was isolated, a preparation was obtained that had a broad band at  $6.07 \mu$ , indicative of an N-acetyl group. However, no conclusion can be drawn, since the preparation was crude. An attempt was made to prepare an acetyl derivative of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid, using the same reaction conditions employed for the preparation of acetylviomycidine. Only starting material could be

isolated from the reaction mixture.

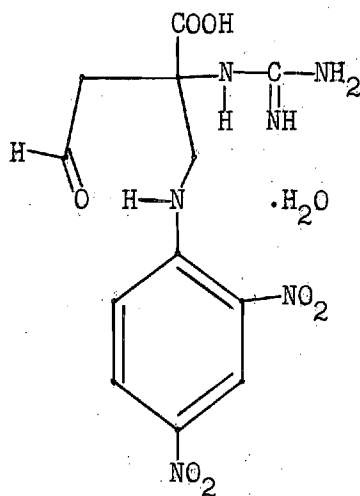
The oxidative degradation of acetylviomycidine was attempted; the anticipated reaction sequence was formulated as follows:



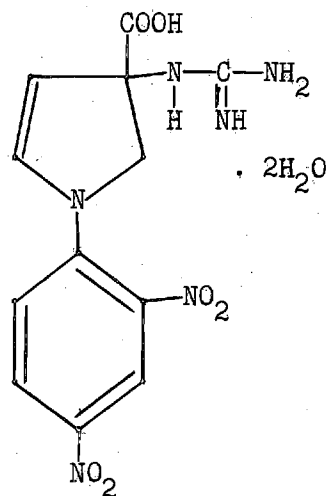
When this sequence of reactions was carried out it was found that the material obtained after the treatment of acetylviomycidine with ozone, hydrogen peroxide, and acid contained no viomycidine as shown by paper chromatography. Since viomycidine is reasonably stable to acid (6), acetylviomycidine gives only viomycidine on acid hydrolysis, and carboxyl and guanidine groups are not attached by ozone (44), this is good evidence

for a double bond in acetylviomycinidene and therefore in viomycinidene itself. After base hydrolysis, it was found that no  $\alpha,\beta$ -diaminopropionic acid was present as shown by paper chromatography. Since this reaction sequence appears reasonable, this result indicates structure VIII might not be correct for acetylviomycinidene.

It was found that viomycinidene could also be characterized by another derivative. 2,4-Dinitrophenylviomycinidene was prepared by a modification of the method for the preparation of 2,4-dinitrophenylamino acids. The derivative was found to be a highly crystalline yellow solid, m. p.  $171.5-172.5^\circ$ . Guanidines are not converted into 2,4-dinitrophenyl derivatives by this reaction (17) so the derivative is probably of a type structurally similar to acetylviomycinidene. Elemental analysis indicated the formula  $C_{12}H_{12}N_6O_6 \cdot 2H_2O$ . The infrared spectrum (Figure 5) was poorly resolved but showed a strong band at  $5.92\ \mu$  and did not show a band at  $3.6\ \mu$ , anticipated for an aldehydic C-H. The analytical sample was not dried to constant weight so it is not known if the water is present as water of hydration or is incorporated covalently into the molecule. Two structures which satisfy the analytical data are IX and X.



IX

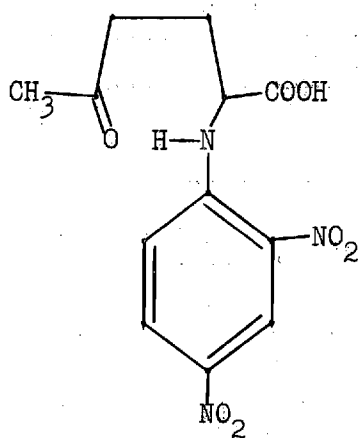


X

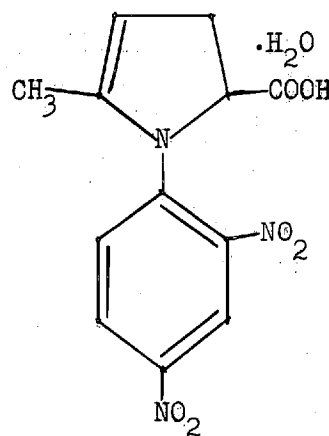


While a structure cannot be assigned to 2,4-dinitrophenylviomycinide, this derivative does serve as a convenient means of characterizing viomycinide.

In connection with this work it was found that a 2,4-dinitrophenyl derivative of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid could also be prepared. Analytical data indicated the formula  $C_{12}H_{13}N_3O_7$ . A carefully dried sample showed a peak at  $2.96 \mu$  in the infrared spectrum. This is the region for N-H stretching vibrations and the absorption in this region indicates that an open-ring compound has been formed. The probable structure of this derivative is XI rather than XII since XI would have an N-H and XII would not. In addition, the infrared spectrum shows a peak at  $5.79 \mu$ , indicative of a ketone, and  $5.93 \mu$ , indicative of a carboxylic acid. These data also indicate XI rather than XII.



XI



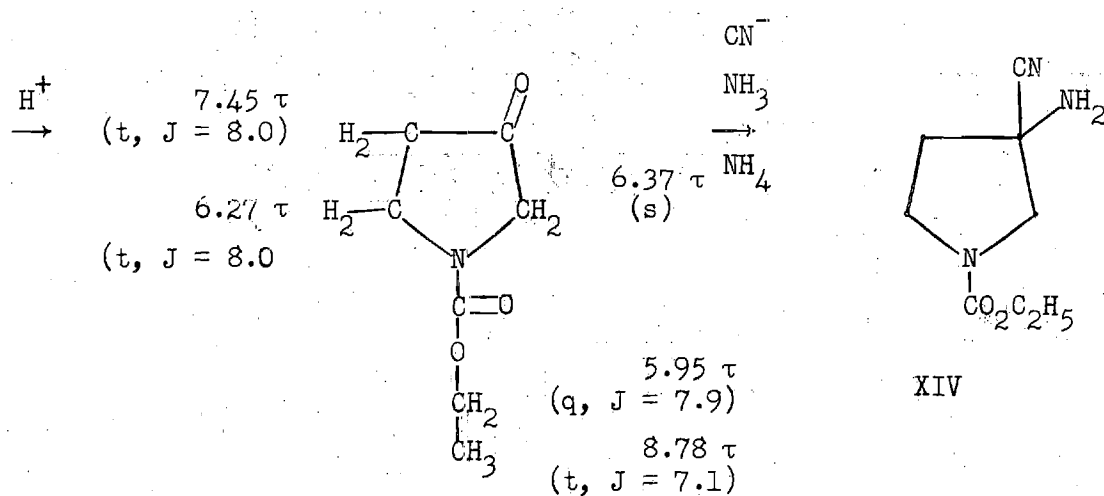
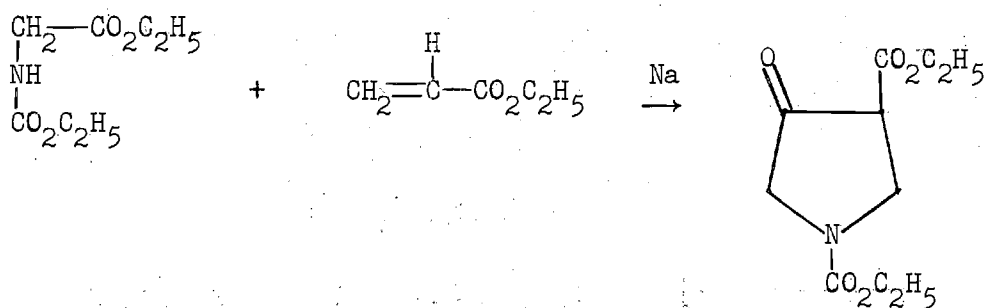
XII

As a  $\Delta^1$ -pyrroline ring was indicated to be present in viomycinide, it was felt that reduction to give a pyrrolidine might result in a substance which might be degraded to a known substance more easily

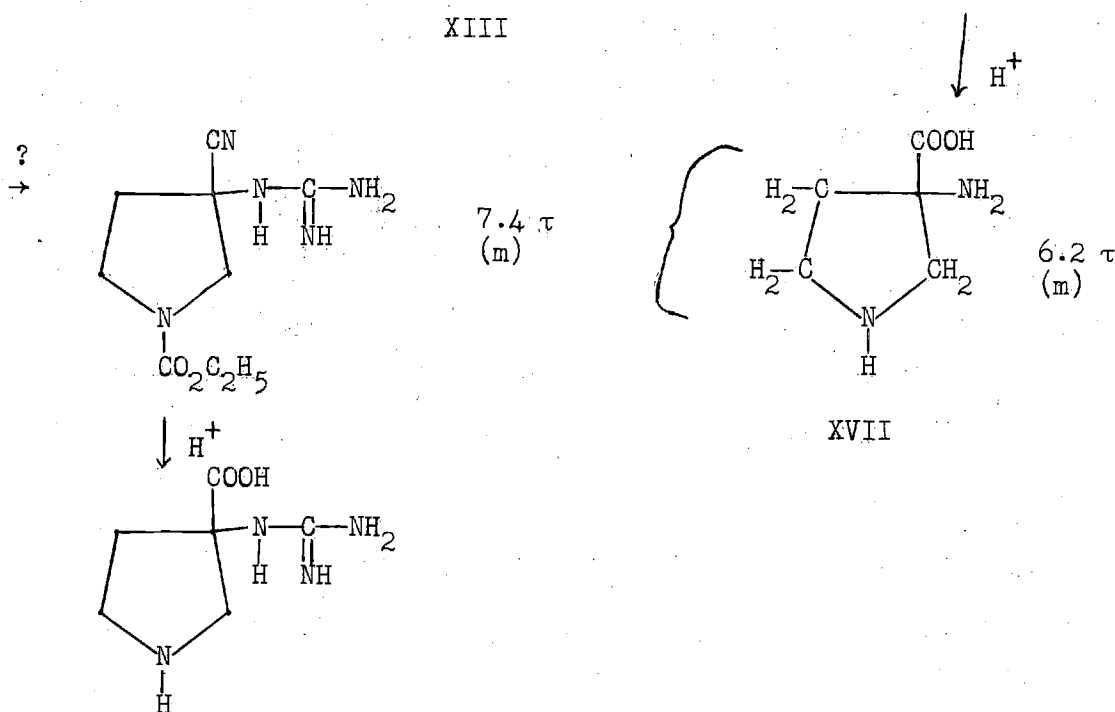
than viomycin. Also, the synthesis of pyrrolidines is simpler than the synthesis of  $\Delta^1$ -pyrrolines. Thus it was thought possible to synthesize any pyrrolidine obtained by reduction of viomycin. Hayes reported a crude dihydroviomycin preparation which was shown to be a mixture by potentiometric titration. A molecular weight of 209 was assumed and the  $pK_a$  values with the number of equivalents of each group is as follows:  $< 3.5$ , (unknown); 5.7, (0.26); 7.9, (0.38); 9.3, 0.22); 11.0, (0.11); 13.4, (0.5) and 13.5-14.0, (0.5). This titration was done using 66 per cent dimethylformamide. The material was optically active,  $[\alpha]_D = + 8.2^\circ$ , indicating that the reduction products are optically active. If the sign of rotation had been negative as in viomycin then the activity might have been due to residual viomycin (8). An unsuccessful attempt was made in this work to obtain pure dihydroviomycin, using rhodium as a catalyst rather than platinum as used by Hayes. Viomycin absorbed about one mole of hydrogen over a period of four days. This preparation was optically active,  $[\alpha]_D = + 8.6^\circ$ . It was found that guanidinoacetic acid was not hydrogenated under the conditions used for the hydrogenation of viomycin. It is possible that hydrogenolysis was taking place during the reduction of viomycin since a mixture of compounds was apparently obtained.

It was thought that acetylviomycin might possibly be more readily reduced than viomycin since N-carbethoxypyrroles are easier to reduce than free pyrroles (45). Also, the acetyl group might decrease or eliminate the possible hydrogenolysis observed with viomycin. With this in mind an attempt was made to synthesize dihydroviomycin.

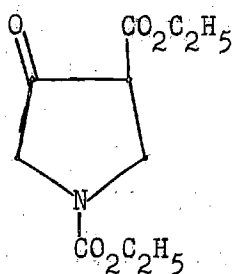
The planned reaction sequence was as follows.



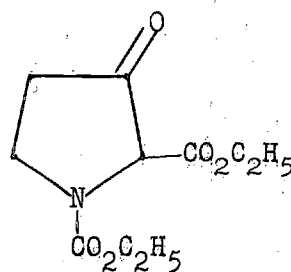
XIII



The numbers on the structures above refer to data obtained from nuclear magnetic resonance spectra of these compounds as evidence for their correct structure. The small letters refer to the multiplicity of the peaks (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and pm = poorly resolved multiplet); the coupling constant,  $J$ , is given in cycles per second. The reaction of N-carbethoxyglycine ethyl ester with ethyl acrylate has been shown to give XV rather than XVI (46).



XV

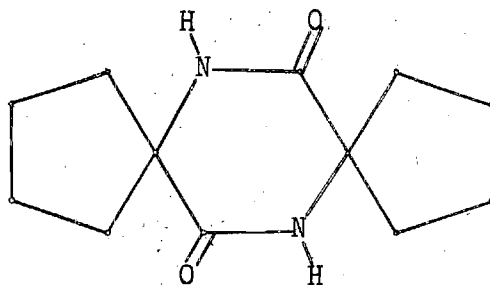


XVI

However, this is not important since in the next step the  $\beta$ -ketoester was hydrolyzed and decarboxylated to give N-carbethoxy-3-pyrrolidone (XIII) (25). The Strecker reaction with this ketone was examined to determine the best reaction time. It was found that the strongest nitrile band ( $4.5 \mu$ ) in the infrared spectra was present for a reaction time of nine days. In this time the largest weight of basic fraction was also obtained. The basic fraction probably contained 3-cyano-3-amino-1-carbethoxypyrrolidine (XIV) as the main constituent since acid hydrolysis of the basic fraction resulted in the formation of 3-aminopyrrolidine-3-carboxylic acid (XVII). Elemental analysis, the infrared spectrum, the  $pK_a$  values (6.5 and 10.1), and the nuclear magnetic resonance spectrum were consistent with the assigned structure for this new amino acid. The amino acid was further characterized by two highly crystalline derivatives:

the N, N'-dibenzoyl-, m. p. 223-225°, and the di-p-hydroxyazobenzene-p'-sulfonate salt, m. p. 256-260°.

As a model compound, attempts were made to convert 1-aminocyclopentanecarboxylic acid to the corresponding guanidino-acid. In the reaction of cyanamide with this amino acid the guanidine derivative was not obtained but the diketopiperazine XVIII was obtained in 8 per cent yield. The elemental analysis and infrared spectrum were consistent with this assignment.



XVIII

Attempts to prepare the guanidine derivative of the aminonitrile (XIV) were initially unsuccessful.

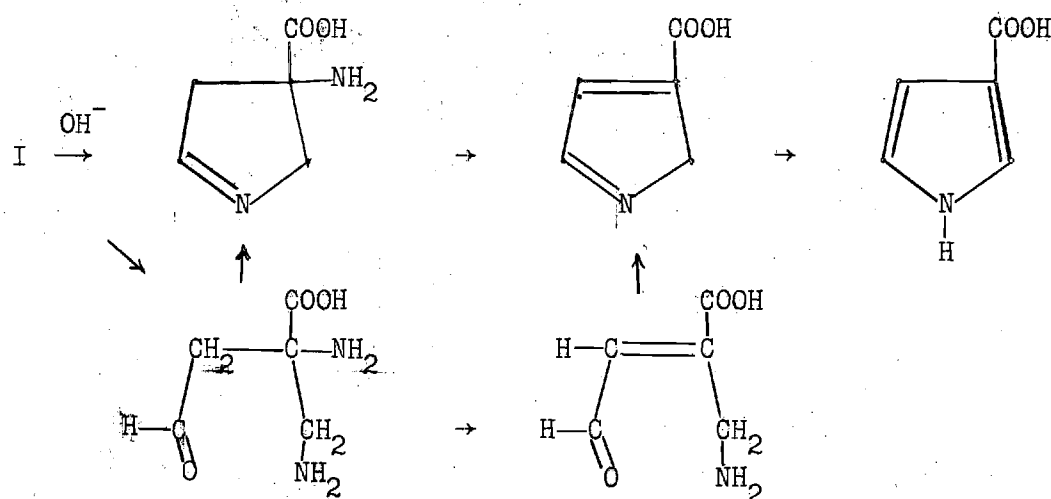
Other results obtained while the above synthetic sequence was in progress obviated the necessity of continuing the reaction sequence, so attempts to convert the amino group of the aminonitrile (XIV) were abandoned.

It had previously been shown that three moles of volatile base and one mole of carbon dioxide were produced per mole of viomycin when viomycin was hydrolyzed with hot aqueous barium hydroxide solution. Arginine gives two moles of volatile base under the same conditions (6). The normal reaction for guanidine compounds with base is:



Therefore, one nitrogen in addition to the two which would normally be produced from a guanidine group is labile to base in viomycin.

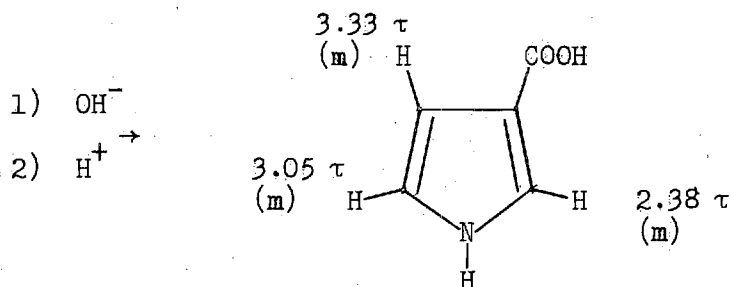
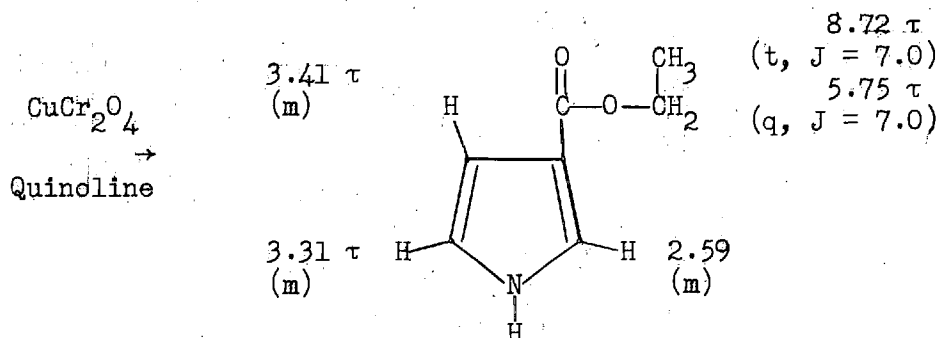
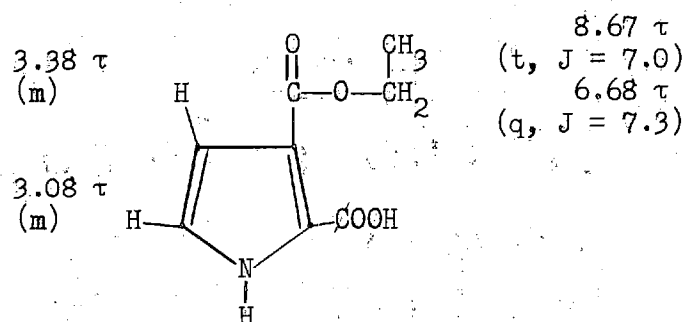
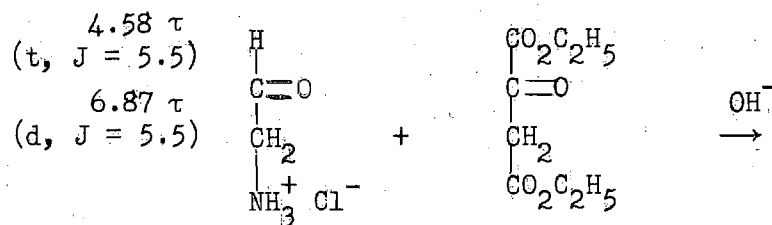
The path for the loss of the third nitrogen atom from viomycin, which is seen to result in the formation of pyrrole-3-carboxylic acid as well, is postulated as follows:



In order to obtain an authentic sample for comparison purposes, pyrrole-3-carboxylic acid was prepared by the following method which had been previously reported (29):

The numbers on the structures below refer to data obtained for nuclear magnetic resonance spectra of these compounds as evidence for their correct structures. The small letters refer to the multiplicity of the peaks (s = singlet, d = doublet, t = triplet, q = quartet,

m = multiplet and pm = poorly resolved multiplet); the coupling constant, J, is given in cycles per second.



The  $\tau$  value for the aldehydic proton of aminoacetaldehyde in acid solution is much higher than that usually observed for aldehydes (ca. 0.35  $\tau$ ). The multiplicity is reasonable for this assignment; however,

the peak position might be explained better on the basis that amino-acetaldehyde exists in solution as the hydrate. This is not unreasonable since the electron withdrawing group on the adjacent carbon atom would stabilize the hydrate just as the trichloromethyl group in chloral hydrate stabilizes the hydrate.

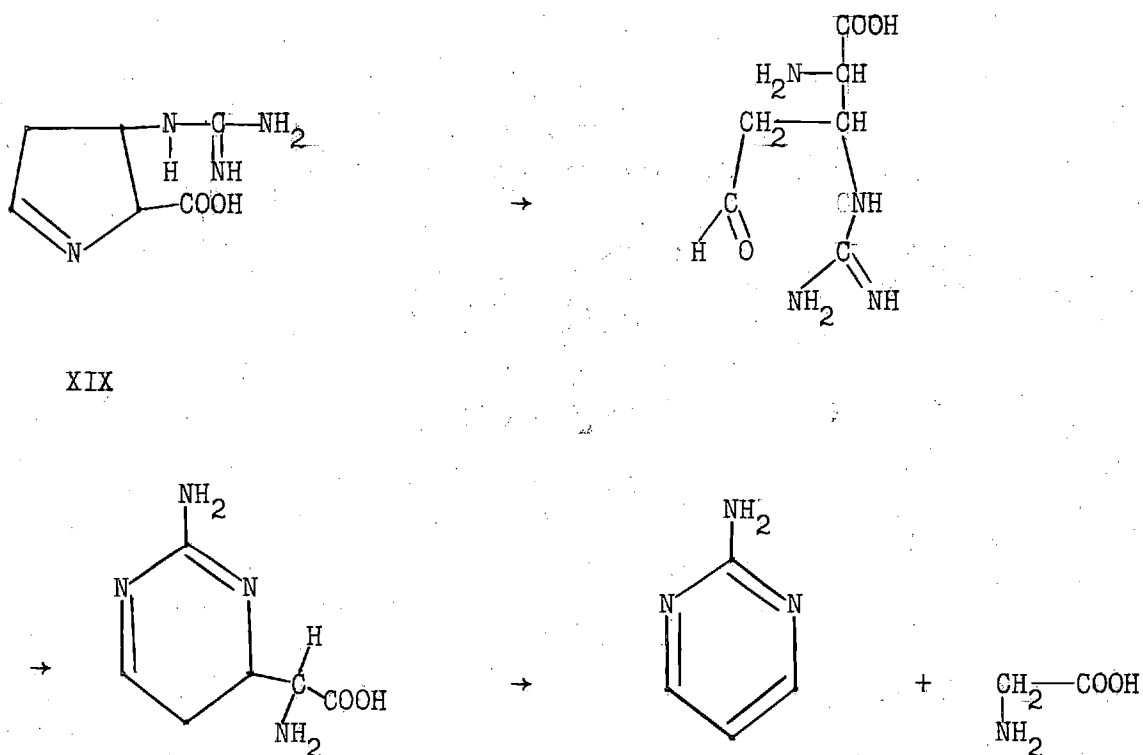
The assignments in the above pyrroles are made as stated since the lowest absorptions in pyrrole, furan, and thiophene are due to the protons on the carbon atoms adjacent to the heteroatom (47).

Viomycin was hydrolyzed in hot barium hydroxide solution and the hydrolysate was passed over an IRC 50 ( $H^+$ ) column. The eluate from this column would contain only acidic and neutral compounds. From this eluate there was obtained a pyrrole acid in 22 per cent yield. This compound and pyrrole-2-carboxylic acid were found to have identical infrared, ultraviolet, and nuclear magnetic resonance spectra, the same  $R_F$  value and the same color when the chromatograms were sprayed with Ehrlich reagent. The melting point of authentic pyrrole-2-carboxylic acid varied with the rate of heating so this was judged to be a poor test for comparison. The melting behavior was similar for the two, however, and quite different from the melting behavior of pyrrole-3-carboxylic acid. Pyrrole-3-carboxylic acid was shown to be different from authentic pyrrole-2-carboxylic acid and the pyrrole-2-carboxylic acid from viomycin by all of the criteria listed above.

The formation of pyrrole-2-carboxylic acid from viomycin shows that in viomycin there are five carbon atoms in a straight chain with the carboxylic acid group at one end of the chain. This powerful evidence is quite adequate to eliminate structure I from consideration as





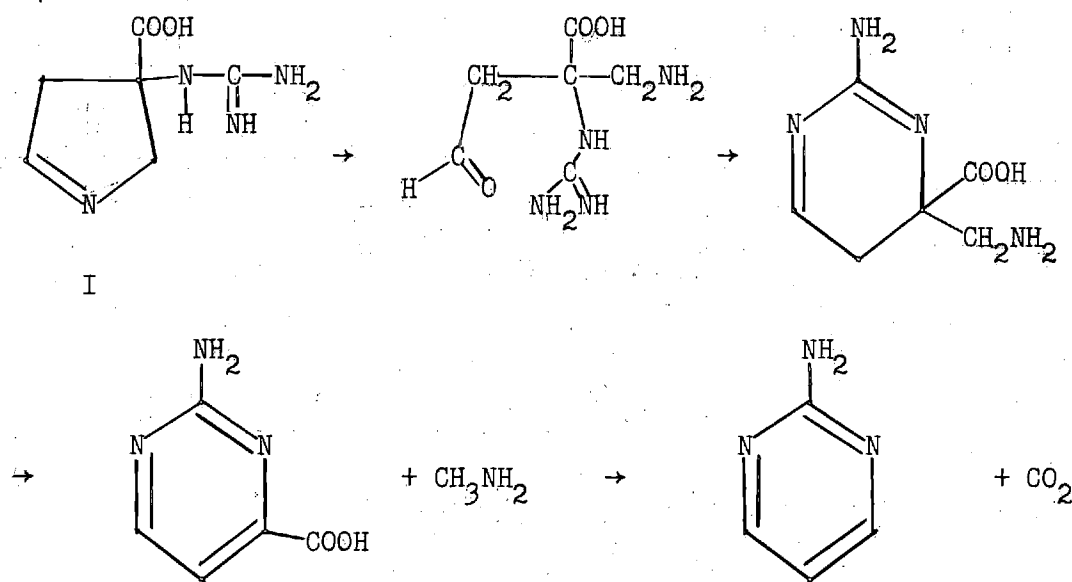


The 2,4-dinitrophenyl derivative of this preparation was prepared and a yellow crystalline compound was obtained. It had the same melting point as authentic 2,4-dinitrophenyl-glycine, the same  $R_F$  values in three solvent systems and the same absorption peaks in the infrared spectrum. These data prove the formation of glycine from viomycin on hydrolysis in concentrated sodium hydroxide. Less than one milligram of the derivative was obtained, indicating that about the same amounts of glycine and 2-aminopyrimidine were produced.

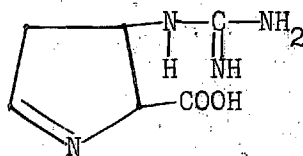
In order to obtain the glycine the hydrolysate had been passed over an IRC 50 ( $H^+$ ) column and the eluate evaporated to dryness. It was found that the material contained in a benzene extract of this residue contained about 1 mg. of pyrrole-2-carboxylic acid as evidenced by the

ultraviolet spectrum. Thus it is possible that both reaction pathways are operating in both the sodium hydroxide and the barium hydroxide reactions.

A nuclear magnetic resonance spectrum in water of the hydrochloride of the volatile bases from the barium hydroxide hydrolysis of viomycin revealed that no aliphatic amines were present because no absorption was present above a  $\tau$  value of 5. For example methylamine, ethylamine and *n*-propylamine have absorptions above 5  $\tau$ . The spectrum did show two sharp singlets at 1.95  $\tau$  and 2.75  $\tau$ . This material is neither pyrrole nor 2-aminopyrimidine, since the nuclear magnetic resonance spectra of these compounds are quite different from that of the unknown. Pyrrole in water shows two quartets of 2.94  $\tau$  and 3.62  $\tau$  while 2-aminopyrimidine in dilute hydrochloric acid shows a doublet of 1.24  $\tau$  and a triplet at 2.95  $\tau$ . The absence of methylamine is added evidence against structure I, since the mechanism for the formation of 2-aminopyrimidine from I would predict the formation of methylamine in the following way:



The formation of pyrrole-2-carboxylic acid from viomycidine places the carboxylic acid group of viomycidine at the 5 position on the  $\Delta^1$ -pyrroline ring. The formation of 2-aminopyrimidine places the guanine group at the 4 position and the formation of glycine lends support to both of these assignments. From these data it appears that viomycidine must be formulated as 4-guanidino- $\Delta^1$ -pyrroline-5-carboxylic acid, XIX.



XIX

A Discussion of the Nuclear Magnetic Resonance Spectra of Viomycidine and Related Compounds: Possible Structures for Viomycidine.-- The nuclear magnetic resonance spectrum of viomycidine hydrochloride in deuterium oxide solution revealed a peak at 7.43  $\tau$  (two protons, an unsymmetrical singlet), a peak at 5.38  $\tau$  (two protons, a sharp singlet), and a broad peak at 4.37  $\tau$  (one proton, a poorly resolved multiplet). Essentially the same data were obtained over a period of three years from spectra of several separate solutions. In order to confirm the number of protons in each peak a spectrum of viomycidine hydrochloride in deuterium oxide solution was determined near the end of this research. The solution was deoxygenated so as to minimize paramagnetic broadening of peaks by dissolved oxygen. The ratio of protons was confirmed but it was found that the peak at 7.43  $\tau$  was clearly split into a triplet ( $J = 1.9$  cps.). This spectrum is given as Figure 2.

The nuclear magnetic resonance spectrum of viomycin hydrochloride in trifluoroacetic acid solution revealed the same proton ratio (2:2:1) for the hydrogens bonded to carbon. Each of these peaks was at a slightly lower  $\tau$  value than the corresponding peak in deuterium oxide solution. The peak positions were 7.23  $\tau$ , 5.05  $\tau$ , and 4.07  $\tau$ ; no splitting of any of the peaks was observed. Three additional absorption peaks were observed at 3.00  $\tau$  (two protons), 1.98  $\tau$  (one proton), and 1.48  $\tau$  (one proton); none of these peaks was split. This spectrum is given as Figure 1.

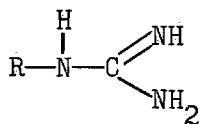
Among the several facts which must be explained in the observed spectra of viomycin are the number of protons which appear in the trifluoroacetic acid solvent spectrum but not in the deuterium oxide solvent spectrum. As the proton of the carboxylic acid group would enter rapid chemical exchange with trifluoroacetic acid, the four additional proton absorptions observed must be due to protons attached to nitrogen.

An accumulation of spectral data indicates that amino compounds are not protonated in trifluoroacetic acid solution. The spectra of a number of amino acids, guanidines, guanido acids, and aliphatic and aromatic amines in trifluoroacetic acid have been determined. The ratio of N-H absorptions to C-H absorptions was determined by spectral integrations and also by weighing the area enclosed by the peak. In essentially all cases, the number of N-H protons observed corresponded exactly to the unprotonated form of the basic compound present. Trifluoroacetic acid would be expected to be a poor ion-solvating medium as it would be expected to have a dielectric constant comparable to that of acetic acid (ca. 6). Consequently the absence of protonation does not seem unreasonable. In fact, glacial trifluoroacetic acid has been found in these

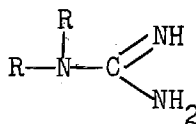
studies to act as a strong base because amino acid zwitterions and guanidinium salts are deprotonated by it.

The nuclear magnetic resonance spectrum of  $\Delta^1$ -pyrroline in trifluoroacetic acid solution (Figure 7) shows absorptions characteristic only of C-H groups; no N-H absorption was observed which might be expected for the protonated form of the imine. In addition, the nuclear magnetic resonance spectrum of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid in trifluoroacetic acid solution shows no peaks that are not present in the spectrum of a deuterium oxide solution of the compound, showing that this  $\Delta^1$ -pyrroline also is not protonated in trifluoroacetic acid solution.

Thus the N-H absorption peaks in the trifluoroacetic acid spectrum of viomycin must be ascribed to the guanidine protons present. The nuclear magnetic resonance spectra of a number of guanidines indicate not only that these compounds are not protonated in trifluoroacetic acid solution but that chemical exchange between the guanidine protons is retarded so that magnetically different protons absorb at different frequencies. In summary of data obtained on a number of compounds, it may be stated that mono-substituted guanidines (XX) have a broad three-proton absorption and a one-proton absorption peak (usually sharper), while N,N-disubstituted guanidines (XXI) have only a broad three-proton absorption peak.

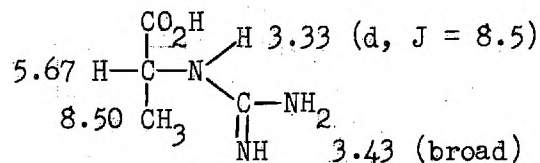


XX

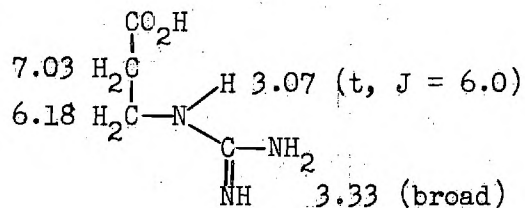


XXI

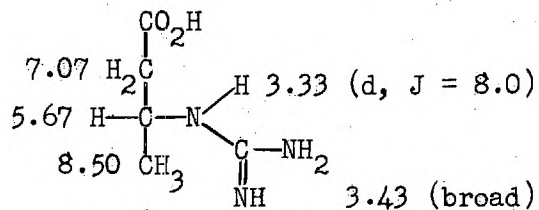
Coupling of the N-H groups to adjacent C-H groups has been observed to follow the ordinary multiplicity rules. Some of these data (48) are given by the formulas for  $\alpha$ -guanidopropionic acid (XXII),  $\beta$ -guanidopropionic acid (XXIII), and  $\beta$ -guanidobutyric acid (XXIV) which follow:



XXII



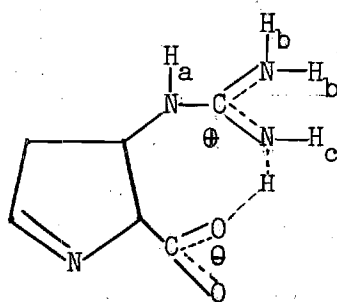
XXIII



XXIV

The spectrum of viomycin in trifluoroacetic acid indicates that there are three distinct types of guanidine protons present in a ratio of 1:1:2. Since the guanidine function in viomycin has been shown to be mono-substituted, the observation of three, rather than two types of magnetically different guanidine protons requires an explanation. An examination of a Dreiding molecular model of viomycin (4-guanido- $\Delta^1$ -pyrroline-5-carboxylic acid) reveals that whether the guanidine or

carboxyl groups are cis- or trans-oriented, the guanidine protons are in quite close proximity to the carboxyl group (XXV). In formula XXV, it is seen that there would be three distinct types of guanidine protons present (a, b, and c), giving rise to the observed individual peaks (ratio of protons, 2:1:1).



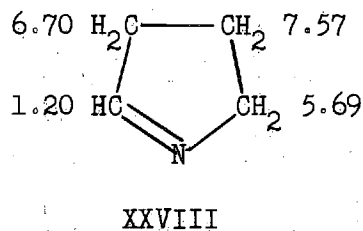
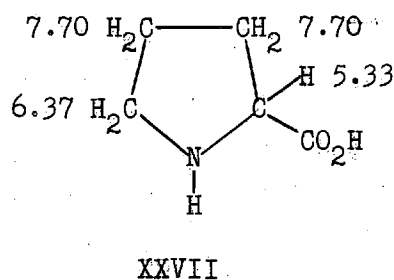
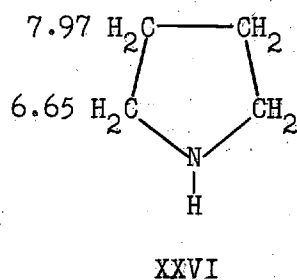
XXV

Only if this hydrogen-bonded structure were very stable would the guanidine protons b not be equivalent to the guanidine proton c. This would require that rotation would be so slow that the shielding experienced by protons b and c would not be averaged to a single value. Unfortunately an appropriate model compound (cis- or trans-2-guanidocyclopentanecarboxylic acid), in which the guanidine protons might show individuality in trifluoroacetic acid solution, is not available.

In addition to the individuality shown by the guanidine protons it is also necessary to discuss the positions of absorptions of the protons bound to carbon. In particular, it is necessary to show that it is not unreasonable for the protons at positions 4 and 5 of the  $\Delta^1$ -pyrroline ring to have the same  $\tau$  value (5.05) and that it is not unreasonable that these protons are not split by one another. An attempt will be made



to estimate a value for each proton on the basis of spectra of known model compounds. The  $\tau$  values for the various absorptions of the model compounds were assigned on the usual bases, that is, observed multiplicities of coupled interactions, intensities observed from spectral integrations, and by using the fact that the more "hydrocarbon-like" a given group is, the more shielded it is. Thus, a methylene group adjacent only to methylene groups has a higher  $\tau$  value than a methylene group which is adjacent to a nitrogen or oxygen atom. As a test of the method, it was thought desirable to estimate some of the absorptions of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid using pyrrolidine (XXVI), proline (XXVII), and  $\Delta^1$ -pyrroline (XXVIII) as model compounds. The absorption values in trifluoroacetic acid solution assigned to the proton groups in these compounds are given by the formulas below(48). It may be seen that replacing an

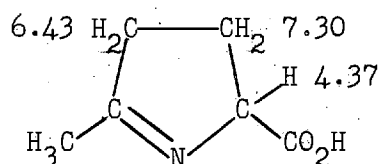


$\alpha$ -proton of pyrrolidine by a carboxyl group changes the  $\alpha$ -proton absorption from 6.65  $\tau$  to 5.33  $\tau$  ( $\Delta = 1.32$  ppm). If

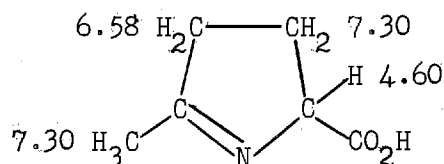
then a proton of the  $C_5$ -methylene group of  $\Delta^1$ -pyrroline were replaced by a carboxyl group, an estimate of the remaining  $C_5$ -proton would be expected to be at  $5.69 - 1.32 = 4.37 \tau$ . The observed value for this proton in 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid is  $4.60 \tau$  in trifluoroacetic acid solvent. The difference in the  $\beta$ -methylene absorption of pyrrolidine and proline is seen to be  $7.97$  and  $7.70 \tau$  ( $\Delta = 0.27$  ppm). The  $C_4$ -methylene group of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid would then be expected to absorb at  $0.27$  ppm less than the absorption of the  $C_4$ -methylene absorption of  $\Delta^1$ -pyrroline ( $7.57 \tau$ ), or at  $7.30 \tau$ . The observed value for these protons in 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid is  $7.30 \tau$ . The  $C_3$ -methylene protons of  $\Delta^1$ -pyrroline absorb  $1.27$  ppm to lower field than the  $\beta$ -protons of pyrrolidine ( $7.97 - 6.70 = 1.27$ ). Therefore the  $C_3$ -methylene protons of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid would be expected to absorb  $1.27$  ppm to lower field than the  $C_4$ -methylene protons of proline, or at  $6.43 \tau$  ( $7.70 - 1.27 = 6.43$ ). The observed value for these protons in 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid is  $6.58 \tau$ . The estimated absorption values for 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid hydrochloride in trifluoroacetic acid solution are given by formula XXIXa; the values determined from the spectrum of the compound in trifluoroacetic acid solution are given by formula XXIXb, and the values determined from the spectrum of the compound in deuterium oxide solution are given by formula XXIXc. It may be seen that the overall agreement of the estimated values and the observed values is reasonably good.

The best model compound available for estimating the absorption positions of viomycin itself is 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid (XXIXb) since the only correction necessary is that due to the

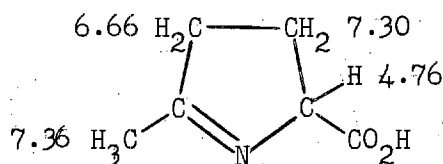
guanidine group at the  $C_4$  position.



XXIXa



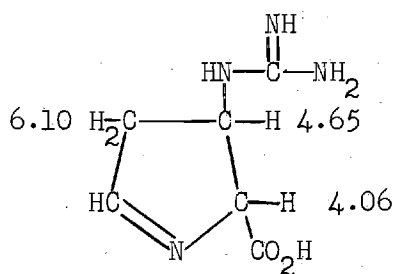
XXIXb



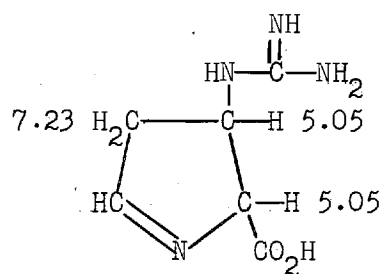
XXIXc

Other compounds which are used in the estimate are butyric acid and  $\beta$ -guanidobutyric acid. The absorption values in trifluoroacetic acid assigned to the proton groups in  $\beta$ -guanidobutyric acid are given by formula XXIV; the absorption positions of butyric acid in trifluoroacetic acid are:  $\alpha$ -CH<sub>2</sub>, 7.61  $\tau$ ;  $\beta$ -CH<sub>2</sub>, 8.32  $\tau$ ;  $\gamma$ -CH<sub>3</sub>, 8.98  $\tau$ . The effect on a methylene group  $\alpha$  to a carboxylic acid group when a guanidine group is substituted on the  $\beta$ -carbon atom is seen to decrease the absorption value by 0.54 ppm (7.61 - 7.07). Thus, substitution of a guanidine group at  $C_4$  of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid should decrease the  $\tau$  value of the proton  $\alpha$  to the carboxylic acid ( $C_5$ ) by 0.54 ppm. This gives an estimated absorption value of 4.06  $\tau$  (4.60 - 0.54) for the  $C_5$  proton of viomycin. The observed value is 5.05  $\tau$ . The effect on a methylene group  $\beta$  to a carboxylic acid group when a  $\beta$ -guanidine group is substituted is to decrease the absorption value by 2.65 ppm (8.32 - 5.67). Therefore the  $C_4$  proton of viomycin would be expected to have

an absorption value 2.65 ppm lower than the  $C_4$  proton of 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid. Thus the estimated value for the  $C_4$  proton of viomycidine is 4.65  $\tau$  (7.30 - 2.65); the observed value is 5.05  $\tau$ . The effect on the methyl group in butyric acid on substituting a guanidine group on the adjacent carbon is to decrease the absorption value by 0.48 ppm (8.98 - 8.50). Therefore the  $C_3$  proton in viomycidine should have an absorption value 0.48 ppm lower than the model compound. Thus the estimated value for the  $C_3$  proton of viomycidine is 6.10  $\tau$  (6.58 - 0.48); the observed value is 7.23  $\tau$ . These estimated values do not taken into account any effect caused by the methyl group at  $C_2$  in 2-methyl- $\Delta^1$ -pyrroline-5-carboxylic acid. The estimated absorption values for viomycidine in trifluoroacetic acid solution are summarized in formula XXXa; the observed values are given in formula XXXb. The only estimated value that is very close to the observed value is that for the  $C_4$  proton.



XXXa



XXXb

However, the estimated values for the  $C_4$  and  $C_5$  protons are reasonably close to each other. The estimated values may be in error because substitution of a guanidine group on a five-membered ring might cause a much different effect than in an open chain compound. The formation of a rigid hydrogen-bonded system might also give an effect which cannot

be estimated. In conclusion, the observed absorption values for viomycin are not unreasonably different from values estimated from known compounds. It is also not unreasonable that the  $C_4$  and  $C_5$  protons of viomycin should have the same absorption value.

The observed multiplicity and lack of multiplicity in the nuclear magnetic resonance spectrum of viomycin must also be rationalized in the discussion of the spectrum. Karplus (49) has predicted on theoretical grounds that the values of coupling constants are proportional to  $\cos^2\theta$ , where  $\theta$  is the dihedral angle between the two C-H bonds concerned. This relationship is in good agreement with the observed values for a number of substituted ethanes and for cyclohexane derivatives (49). A plot of the coupling constant,  $J$ , versus dihedral angle,  $\theta$ , indicates that the coupling constant for substituted ethanes is smallest when the angle is about  $90^\circ$  and is largest when the angle is  $0^\circ$  or  $180^\circ$ . In the equation for calculating the coupling constant several effects besides the dihedral angle are important. For example, bond length and a factor for substituent groups are significant. No study has been made of guanidine compounds or  $\Delta^1$ -pyrroline rings so it is quite difficult to estimate coupling constants for viomycin without a very elaborate mathematical treatment. For example, the  $C_3$  and  $C_5$  protons form  $0^\circ$  dihedral angles with the  $C_4$  proton in  $\Delta^1$ -pyrroline itself. This would indicate large  $J$  values for these two groups but no splitting is observed; only the  $C_4$  protons show multiplicity. The nuclear magnetic resonance spectra of hydroxyproline and allo-hydroxyproline have been completely analyzed (50) and it was found that coupling constants did not necessarily depend on the dihedral angles. For some interactions the coupling constants were

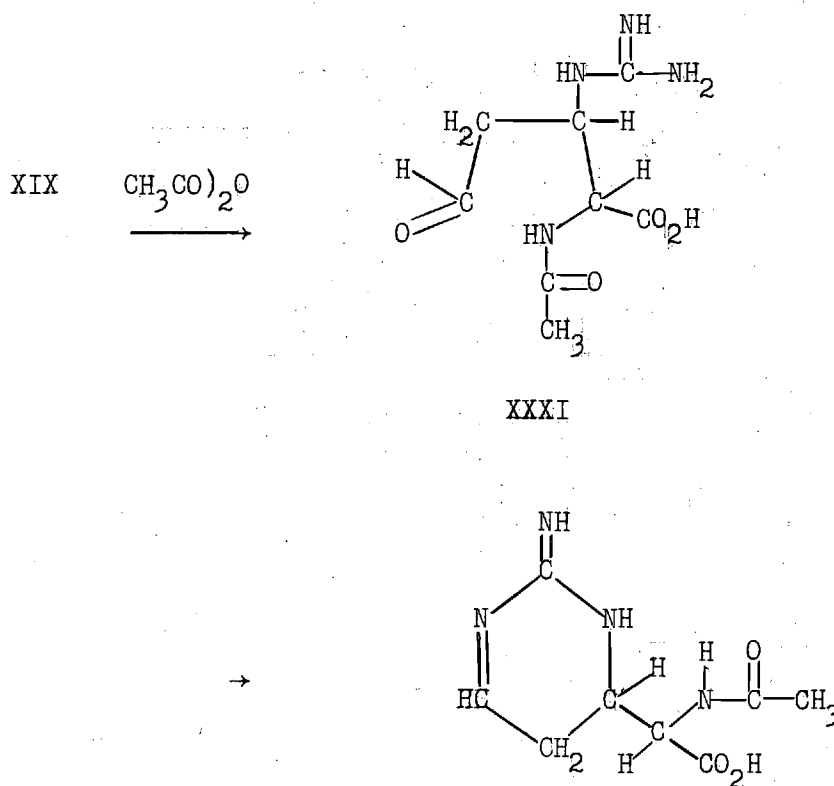
as low as 1 cps even though the angle was essentially  $0^\circ$ . In other groups a coupling constant of 4 - 5 cps was observed even though the angle was not as favorable. Thus, while splitting might be expected for viomycinidine on the basis of the various dihedral angles, lack of multiplicity is not unreasonable.

Based on structure I for viomycinidine, structure VIII was initially assigned for acetylviomycinidine in light of the common reaction of  $\Delta^1$ -pyrrolines, on acylation, to give N-acyl- $\Delta^2$ -pyrrolines. Consideration of all of the properties of acetylviomycinidine dictate that structure VIII is incorrect. Acetylviomycinidine has  $pK_a$  values of 4.86 and 13.0 in 66 per cent dimethylformamide (34). It gives a negative ninhydrin test, a positive Weber test, a negative Sakaguchi test, and a negative *o*-amino-benzaldehyde test. The infrared spectrum of acetylviomycinidine (Figure 3) is complicated but indicates the absence of an aldehydic proton. On acid hydrolysis acetylviomycinidine reverts to viomycinidine as the only observable product. The nuclear magnetic resonance spectrum of acetylviomycinidine in deuterium oxide solution (Figure 4) shows four exchangeable protons and eight non-exchangeable protons. The spectrum shows peaks at 4.30  $\tau$  (0.78 protons), 5.19  $\tau$  (4.26 protons, water), 5.50  $\tau$  (1.96 protons), 7.73  $\tau$  (3.44 protons), and 8.03  $\tau$  (1.57 protons). The number of non-exchangeable protons combined with the negative Sakaguchi test excludes an N-acetyl- $\Delta^2$ -pyrroline structure similar to VIII.

Since the strongly basic group gives a negative Sakaguchi test, the guanidine group must be di-substituted. The weakly acidic carboxylic acid group cannot be  $\alpha$  to a basic group because of previously cited data. The weakly basic group which is present in viomycinidine is absent

in acetylviomycin. Therefore this group must have been acetylated. Acetylation of the guanidine is unlikely (17), but if this did occur, an additional basic center would be present, as acetylguanidine has a  $pK_a$  of 8.3 (51). A disubstituted guanidine and eight carbon-bonded protons require a rearranged structure.

A ring-opened structure XXXI as commonly results from acetylation of  $\Delta^1$ -pyrrolines is excluded on the basis of the elemental analysis and the absence of an aldehydic proton in the nuclear magnetic resonance spectrum. However, such a structure could well be an intermediate in the reaction, cyclization subsequently giving XXXII. A structure such as XXXII satisfies the analytical data, the  $pK_a$  data, the color reactions, and the number of exchangeable protons as indicated by the nuclear magnetic resonance spectrum.



Easy reversion to viomycidine on acid hydrolysis would be expected by hydrolysis of the N-acetyl group and rearrangement to the more stable cyclic system, a  $\Delta^1$ -pyrroline.

The nuclear magnetic resonance spectrum requires some discussion. There are two high field absorptions (7.73  $\tau$  and 8.03  $\tau$ ) containing a total of five protons but they are in a ration of 3.44:1.57. Clearly the peak at 7.73  $\tau$  must contain the acetyl methyl group (the acetyl methyl group of acetylproline and acetylpyrrolidine absorb at 7.40  $\tau$  (trifluoroacetic acid solution) and 8.08  $\tau$  (neat liquid), respectively). This leaves 0.44 protons in this group which, combined with the peak at 8.03  $\tau$  totals 2.0 protons. This high field absorption must be due to protons on carbon adjacent only to carbon atoms. In considering structure XXXII for acetylviomycidine it is seen that the methylene proton group in the ring satisfies this situation. Furthermore they would be the AB part of a complicated ABCD or ABCX system (52) of interacting nuclei. These systems have not been analyzed mathematically (52, 53) but it is quite reasonable to anticipate a complicated interaction for the AB part of the spectrum. Simpler systems (ABC and ABX), depending on the values of  $\nu$  and J, give spectra for the AB protons as complicated as this. The remaining protons would be expected to have similar absorption to viomycidine and the previously mentioned rationalizations of peak positions would apply to acetylviomycidine.

The peak at 4.30  $\tau$  is a poorly resolved multiplet and contains one proton. This peak position is probably due to the olefinic type proton just as in viomycidine itself. The peak is probably broadened by the adjacent methylene group. The absorption at 5.50  $\tau$  contains a



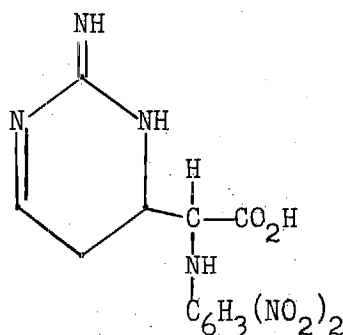
group of peaks. This absorption is probably due to the two methinyl groups which are somewhat similar to the methinyl groups in viomycidine. No good model compound is available for comparison but the peak position would be expected to be somewhat similar to the  $\alpha$ -proton of an N-acetylamino acid. The methylene group of acetylglycine absorbs at  $6.03 \tau$  in deuterium oxide solution and the methinyl proton of acetylproline absorbs at  $5.38 \tau$  in deuterium oxide solution. Acetylglycine is probably a better model. A guanidine group on the adjacent carbon atom should lower the  $\tau$  value so that a value of  $5.50 \tau$  is not unreasonable for this group. The methinyl group which bears a guanidine group would have a low  $\tau$  value because substitution of a guanidine group on a methylene group causes a large decrease in  $\tau$  value. Thus a  $\tau$  value of  $5.50$  is not unreasonable for this group. The observed absorption positions of acetylviomycidine are generally consistent with the proposed structure.

One factor against structure XXXII for acetylviomycidine is the fact that it would ordinarily be expected to have an ultraviolet absorption spectrum. Acetylviomycidine shows only strong end absorption in the ultraviolet region. However, no suitable model compounds are available; no 2-aminodihydropyrimidine is known and no similarly constituted alkylideneguanidine is known. Acetylviomycidine would obviously exist as a zwitterion even in 95 per cent ethanol; its ultraviolet absorption spectrum would be expected to have a lower extinction than the free base.

The properties of acetylviomycidine are reasonably consistent with its formulation as XXXII. As this structure is derived rationally from viomycidine by reactions for which there is adequate precedence,

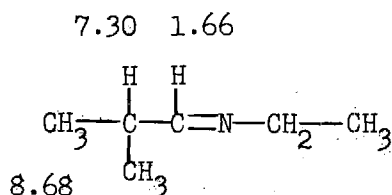
this is taken as added evidence for the correctness of XIX for viomycinine itself.

In connection with this proposal, 2,4-dinitrophenylviomycinine, previously formulated as IX or X, might more correctly be formulated as having structure XXXIII.

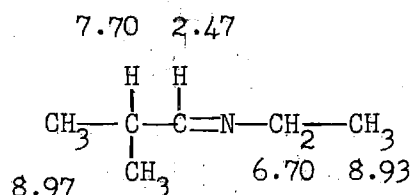


XXXIII

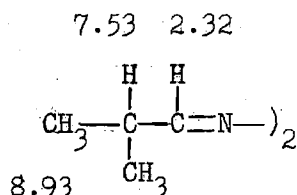
All of the chemical data and the spectral data discussed previously are consistent with the proposed structure for viomycinine. However, the absorption position assigned (4.07  $\tau$  in trifluoroacetic acid solution) to the olefinic proton of viomycinine does not appear to be in agreement with similar protons in model compounds. The observed  $\tau$  value of the olefinic proton of  $\Delta^1$ -pyrroline in trifluoroacetic acid solution is 1.20. The  $\tau$  values observed for *iso*-butylidene-ethylamine in trifluoroacetic acid solution (XXXIVa) and as the neat liquid (XXXIVb) are given below as well as the  $\tau$  values of *iso*-butylideneazine as the neat liquid (XXXV).



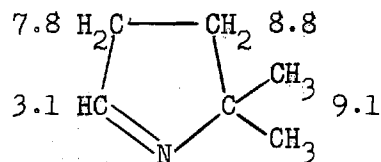
XXXIVa



XXXIVb



XXXV



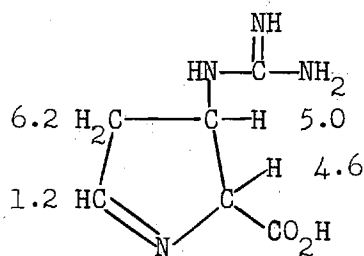
XXXVI

The  $\tau$  values for 5,5-dimethyl- $\Delta^1$ -pyrroline have been reported as the neat liquid (54) and are given by formula XXXVI. Since the absorption value of the olefinic proton in XXXIV decreases 0.81 ppm when the neat liquid is compared to trifluoroacetic acid solution, the estimated absorption value for the  $\text{C}_2$  proton in XXXVI is 2.3  $\tau$  (3.1 - 0.8). The olefinic proton of the azine (XXXV) would be expected to have an absorption value of about 1.5  $\tau$  (2.32 - 0.8) in trifluoroacetic acid solution. In contrast to these data, viomycin does not show absorption in this low field region, the lowest absorption value for a carbon-bonded proton in viomycin being 4.07  $\tau$ .

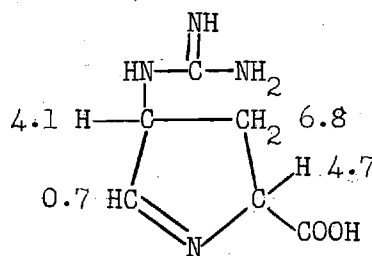
Thus, while structure XIX for viomycin is consistent with all of the chemical evidence and most of the physical evidence, it is difficult to rationalize the absence of a proton absorbing at low field in the nuclear magnetic resonance spectrum. However, it is possible that the presence of a guanidine group and a carboxylic acid group on a  $\Delta^1$ -pyrroline ring might have effects which are at present unpredictable on the peak position of the olefinic-type proton.

The evidence for a  $\Delta^1$ -pyrroline ring in viomycin is strong. However, the placement of the carboxylic acid group and the guanidine

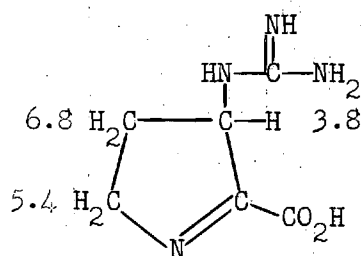
group might be questionable on the basis of the absence of a low field absorption in viomycin. The  $pK_a$  of the carboxylic acid demands that it be on a carbon atom that also bears a nitrogen function. Since the formation of pyrrole-2-carboxylic acid from viomycin dictates that there be five carbon atoms in a straight chain, the carboxylic acid group must be at  $C_2$  or  $C_5$  of the  $\Delta^1$ -pyrroline nucleus. The guanidine group cannot be at either  $C_2$  or  $C_5$  because a very unstable hemi-acetal-type structure would result. Thus all of the possible structures for viomycin are given by formulas XIX, XXXVII, XXXVIII, and XXXIX, exclusive of stereochemical differences.



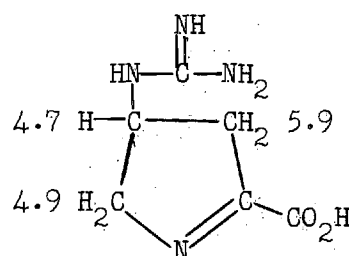
XIX



XXXVII



XXXVIII

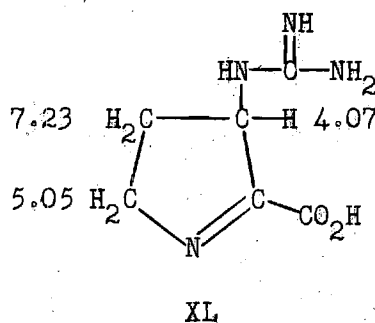


XXXIX

As stated previously, the objection to XIX is the absence of absorption at low field in the nuclear magnetic resonance spectrum indicative of a  $\Delta^1$ -pyrroline which has an olefinic proton. Thus, if XIX is excluded on this basis, XXXVII would also be excluded. In addition,

the formation of glycine and 2-aminopyrimidine is difficult to rationalize on the basis of structure XXXVII for viomycidine.

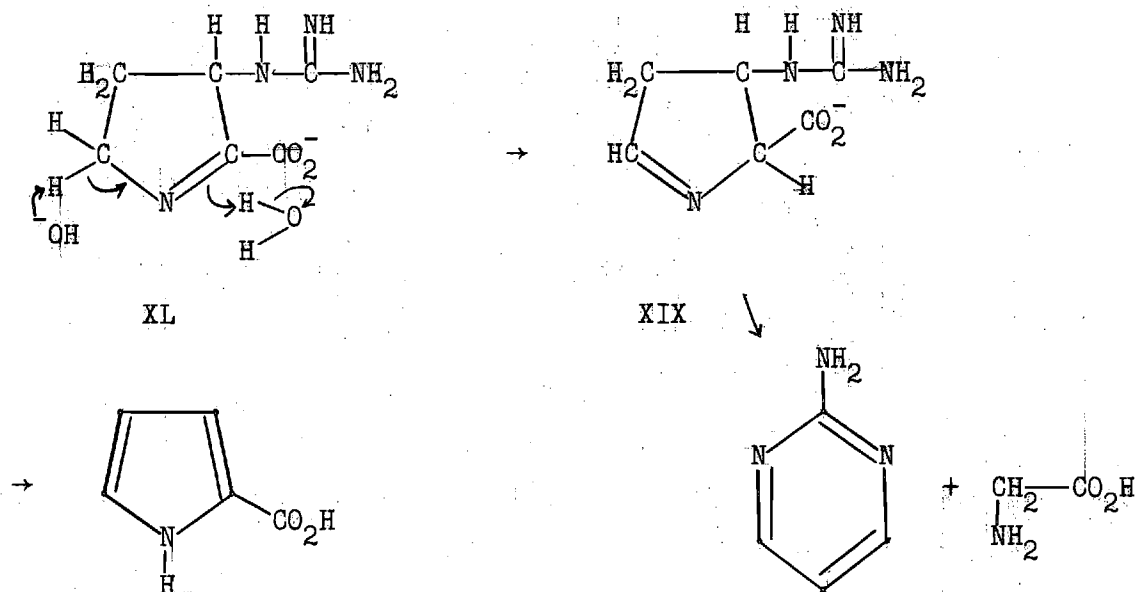
Estimates of the nuclear magnetic resonance peak positions, derived as previously indicated, are given on each of the structures XIX, XXXVII, XXXVIII, and XXXIX. The estimates were made on the basis of peak positions of  $\Delta^1$ -pyrroline, butyric acid,  $\beta$ -guanidobutyric acid, pyrrolidine, and proline. The observed values for viomycidine in trifluoroacetic acid are 4.07 (1H), 5.05 (2H), and 7.23 (2H). Structure XXXIX gives generally poor comparisons; the estimates for structure XXXVIII are closer to the observed values than those for structure XIX. If XXXVIII is the structure of viomycidine the assignment of the observed values in trifluoroacetic acid solution would be as given in XL. This structure obviates



the necessity of having two different methinyl groups with the same  $\tau$  value. Structure XL for viomycidine also permits a hydrogen-bonded cyclic structure so that individuality of the guanidine protons is still possible. Considering all absorptions, structure XL is more consistent with the observed nuclear magnetic resonance spectrum of viomycidine than any of the other three structures.

A rationale of the observed chemistry of viomycidine is more

difficult on the basis of structure XL than on the basis of structure XIX. The path for the formation of pyrrole-2-carboxylic acid, 2-aminopyrimidine, and glycine might be postulated as proceeding through structure XIX. This would involve

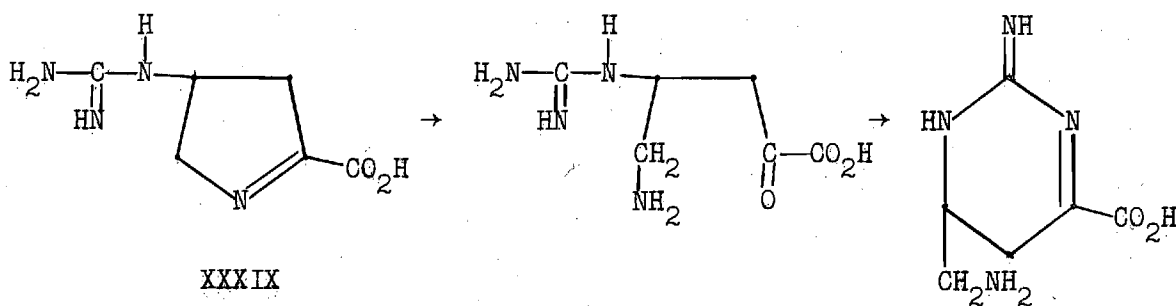


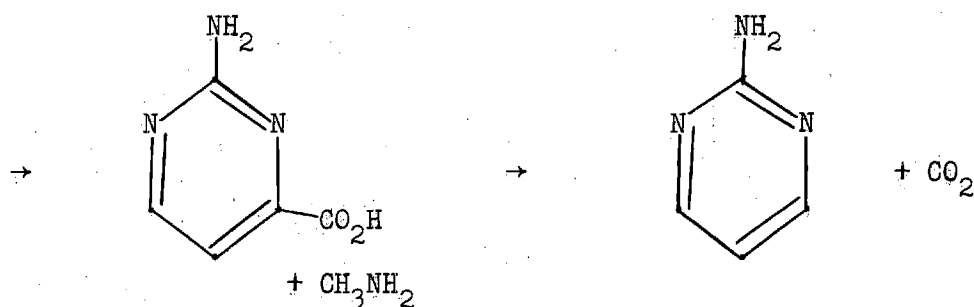
a base-catalyzed isomerization of XL to XIX. The reaction path would then be the same as previously given. It has been reported that some  $\alpha,\beta$ -unsaturated steroidyl-ketones are converted to the  $\beta,\gamma$ -unsaturated ketone in the presence of potassium t-butoxide. The amount of deconjugation varied with the reaction time and with the number of equivalents of base; with one equivalent of base for one hour at room temperature the deconjugation was 40 per cent complete (55). While these ketones are not entirely analogous to XL they do illustrate the isomerization, in alkaline solution, of a double bond out of conjugation. Therefore it is reasonable to expect the two isomers (XL and XIX) to exist in equilibrium in alkaline solution.

Since structure XL is an  $\alpha,\beta$ -unsaturated acid, ultraviolet absorption might be anticipated. However, at no pH is the free carboxylic acid group and the unprotonated amine present in solution. In addition  $\alpha,\beta$ -unsaturated acids have  $\lambda_{\max}$  at very low wavelength (crotonic acid,  $\lambda_{\max}$  204 m $\mu$ ). Thus viomycinine might have maximum absorption in the ultraviolet region but at very short wavelength ( $< 200$  m $\mu$ ). The formation of acetylviomycinine cannot be rationalized on the basis of structure XL for viomycinine except by assuming isomerization to XIX. However, sufficient data are not available to assign the structure of acetylviomycinine with any certainty.

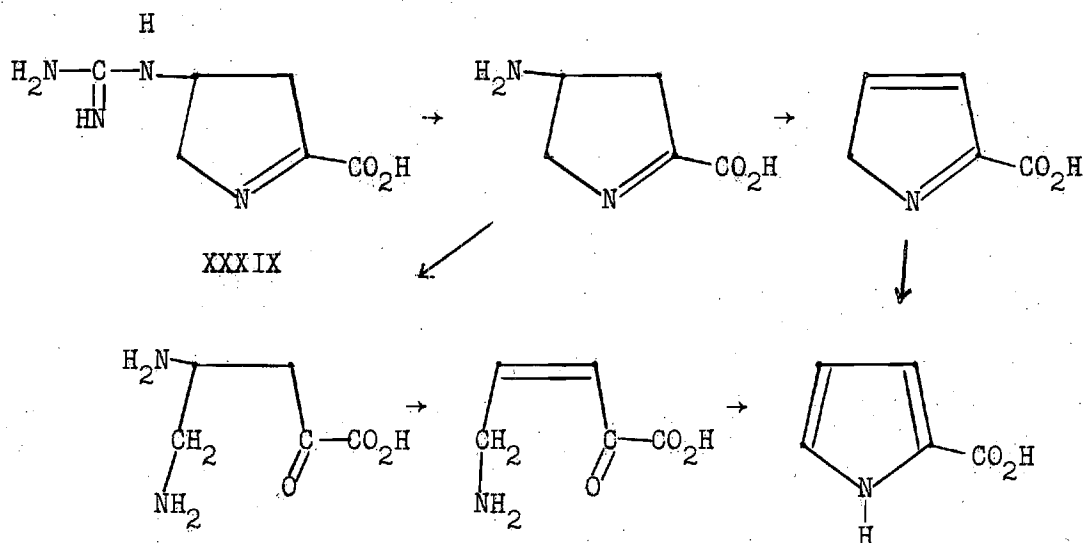
The estimated absorption values for structure XXXIX are not as close to the values observed for viomycinine as the estimated values for structure XXXVII. However, the values are not much worse than the estimated values for XIX. Therefore the nuclear magnetic resonance data are not sufficient to eliminate structure XXXIX from consideration. The chemical evidence is more difficult to explain than on the basis of structure XIX or XXXVII, since isomerization cannot occur to give XIX. Of all structures considered, XIX furnishes the best explanations for the observed chemistry of viomycinine.

The pathway for the transformation of XXXIX to 2-aminopyrimidine is postulated as follows:



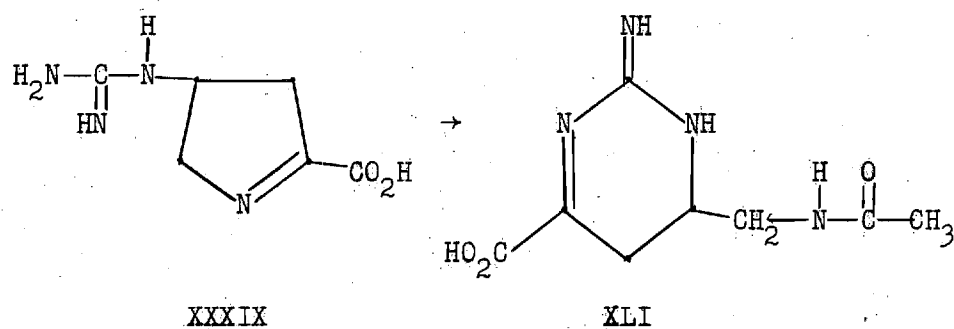


This explanation for the formation of 2-aminopyrimidine is quite reasonable as is the following proposed pathway for the formation of pyrrole-2-carboxylic acid.



No rational mechanism is obvious for the formation of glycine or for the formation of XXXII, the postulated structure for acetylviomycin. A reasonable structure for acetylviomycin, on the basis of XXXIX for viomycin would be XLI, which could be formed in the following way:

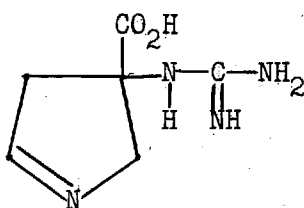




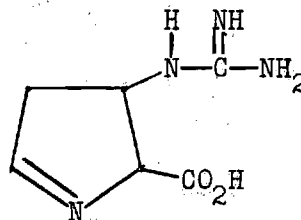
Structure XLI should definitely have an ultraviolet absorption since it contains a system of extended conjugation. The lack of ultraviolet absorption by acetylviomycin, the absence of a rational path for the formation of glycine, and the relatively poor agreement of estimated nuclear magnetic resonance absorption values are considered reasonable evidence that viomycin does not have structure XXXIX.

## CONCLUSIONS

Previously recorded properties of viomycidine have been confirmed. Additional evidence has been obtained which very strongly indicates that viomycidine contains a  $\Delta^1$ -pyrroline ring with guanidine and carboxylic acid functional groups. A previously suggested structure (I) for viomycidine has been rejected by the isolation of pyrrole-2-carboxylic acid from alkaline hydrolysis of viomycidine. This resulted in the proposal of XIX for viomycidine; this proposal was further supported by the isolation of 2-aminopyrimidine and glycine from alkaline hydrolysis of viomycidine.

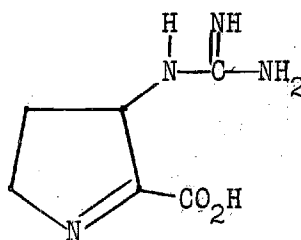


I



XIX

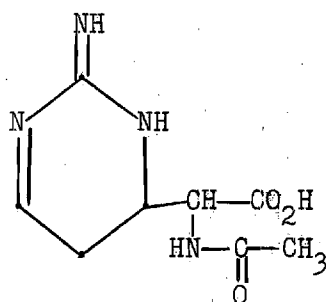
The nuclear magnetic resonance spectrum of viomycidine has been examined critically and has been compared with suitable model compounds. Structure XIX has not been found to be completely satisfactory with these results. An alternate structure, XL, is suggested for viomycidine which is more compatible with the nuclear magnetic resonance results. The chemical data obtained are not as easily rationalized if XL, rather than XIX is the structure, isomerization of XL to XIX during alkaline hydrolysis and acetylation being required.



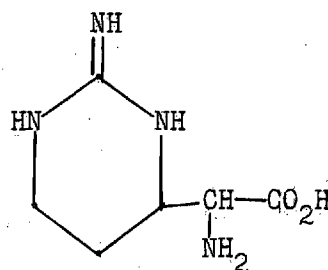
XL

Few additional experiments are directly suggested as a result of this research. It has been shown that no methylamine results on barium hydroxide hydrolysis of viomycin, in which the major product obtained was pyrrole-2-carboxylic acid. Considering structure XL, it would not be unreasonable to anticipate some methylamine to result on strong alkaline hydrolysis. It would be worthwhile to determine the nuclear magnetic resonance spectrum of the volatile bases from a strong sodium hydroxide hydrolysis of viomycin to determine if any methylamine were present.

Viomycin has been shown to yield an acetyl derivative for which structure XXXII is proposed. It has recently been reported (56) that hydrolysis of the antibiotic capromycin produces a guanido amino acid for which, on the basis of limited data, structure XLII is proposed. The remarkable resemblance of this structure to that proposed for acetylviomycin is readily seen. If a small quantity of the amino acid derived from capromycin could be obtained, it would seem worthwhile to reduce some acetylviomycin and subject the product to acid hydrolysis to determine if XLII were produced.



XXXII



XLII

As isomerization of XL to XIX in alkaline solution is postulated, it would be valuable to determine if the nuclear magnetic resonance spectrum of viomycin in deuterium oxide solution changes with time, indicating base-catalyzed exchange. The results would probably be complicated unless isomerization is rapid compared with hydrolysis.

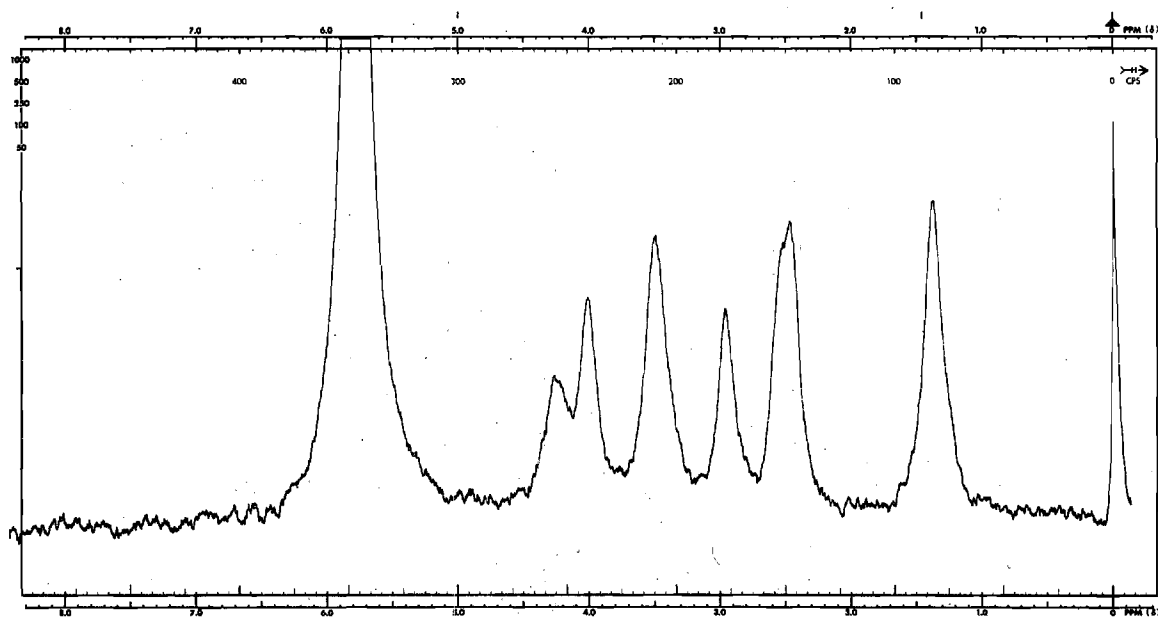


Figure 1. Nuclear Magnetic Resonance Spectrum of Viomycinide Hydrochloride in Trifluoroacetic Acid Solution.

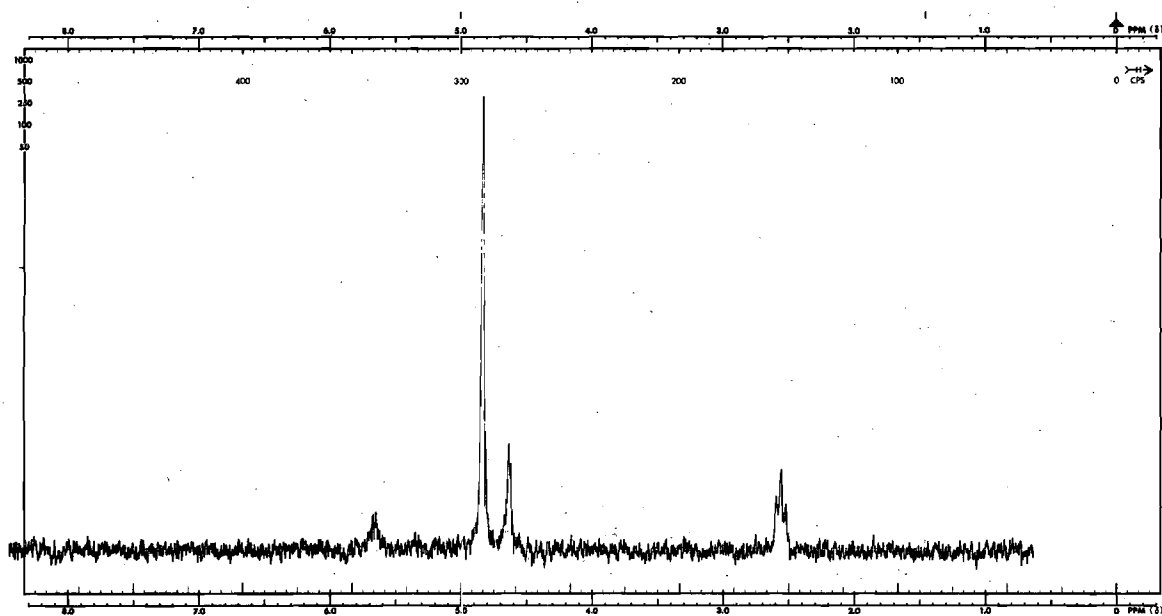


Figure 2. Nuclear Magnetic Resonance Spectrum of Viomycinide Hydrochloride in Deuterium Oxide Solution.

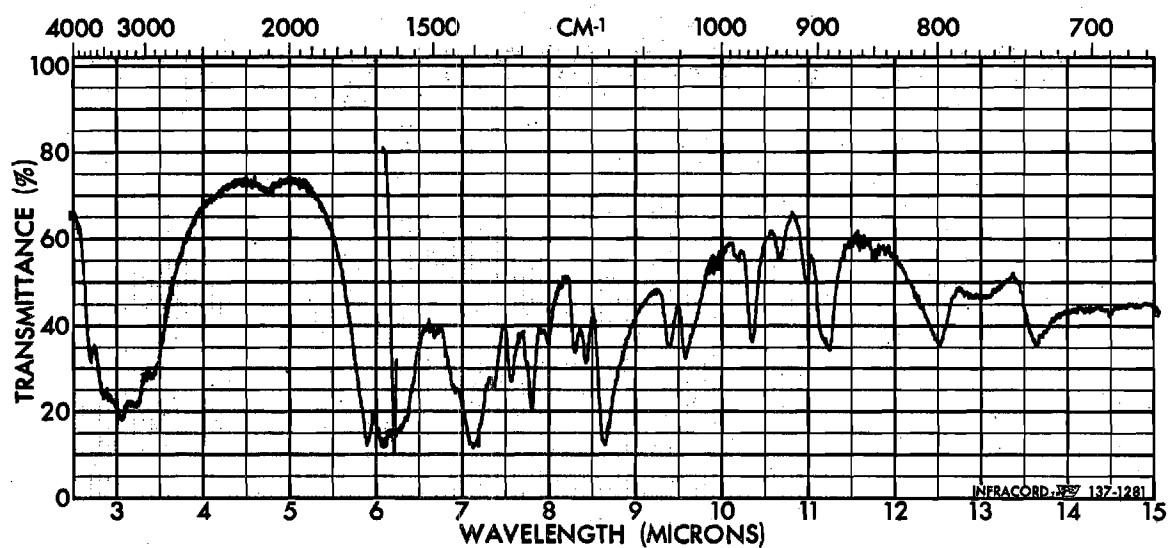


Figure 3. Infrared Spectrum of Acetylviomycin.

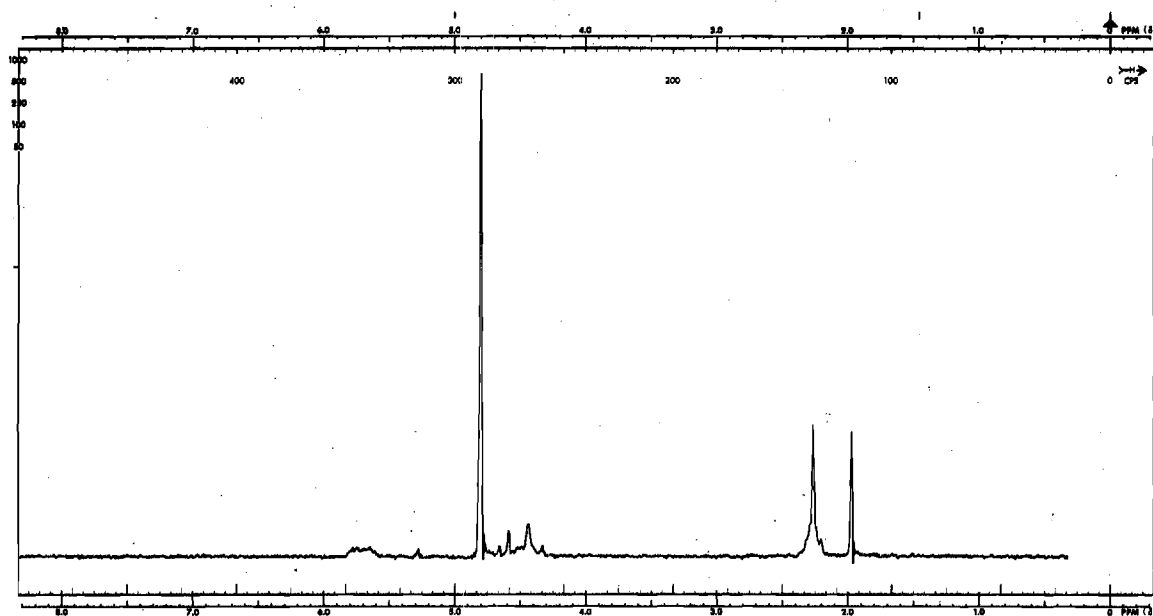


Figure 4. Nuclear Magnetic Resonance Spectrum of Acetylviomycin in Deuterium Oxide Solution.

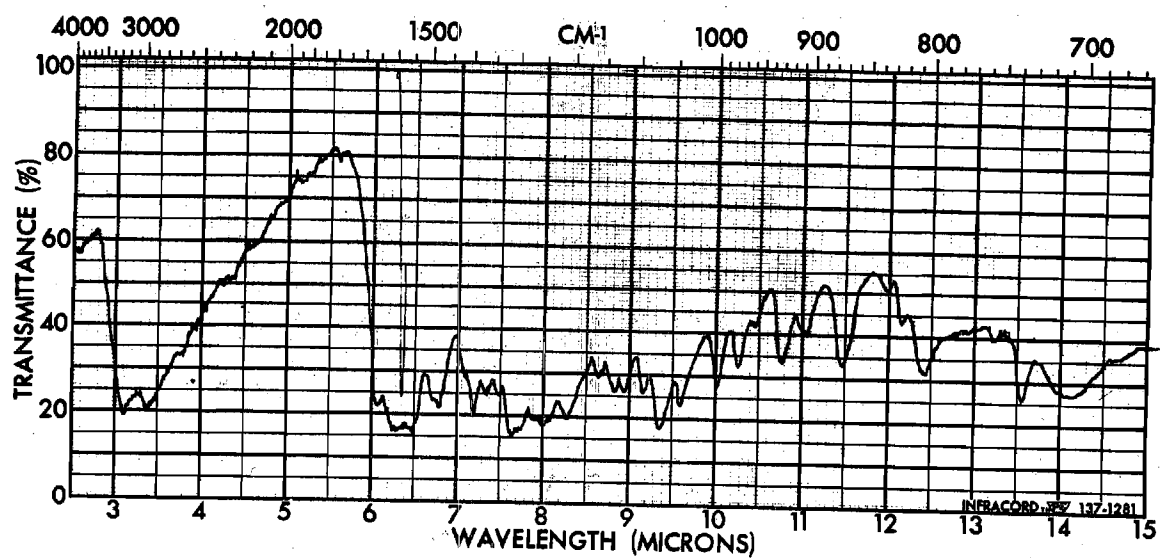


Figure 5. Infrared Spectrum of 2,4-Dinitrophenylviomycinide.

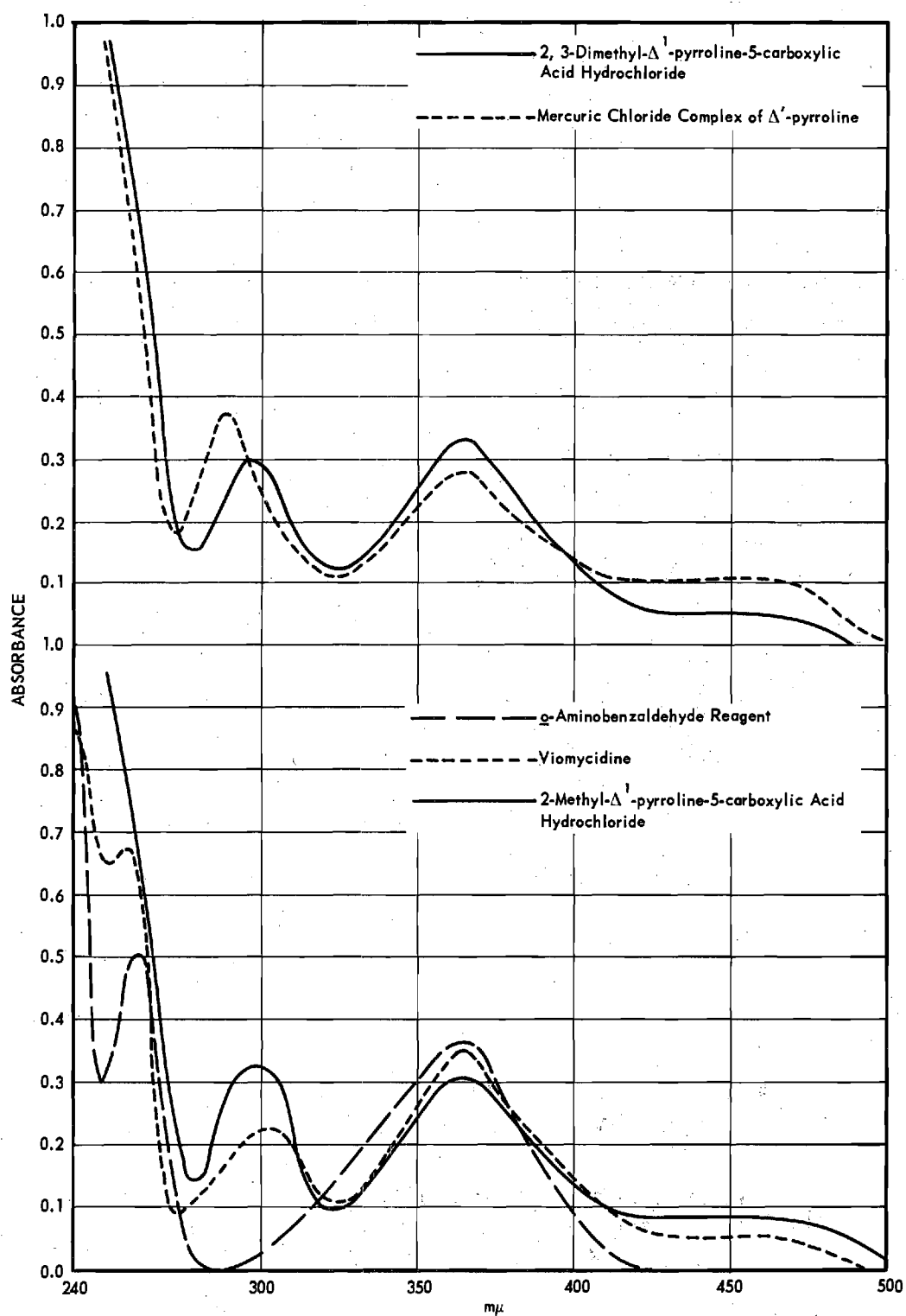


Figure 6. Ultraviolet and Visible Spectra of the Reaction Mixture from some  $\Delta^1$ -Pyrrolines and Viomycin with o-Aminobenzaldehyde.



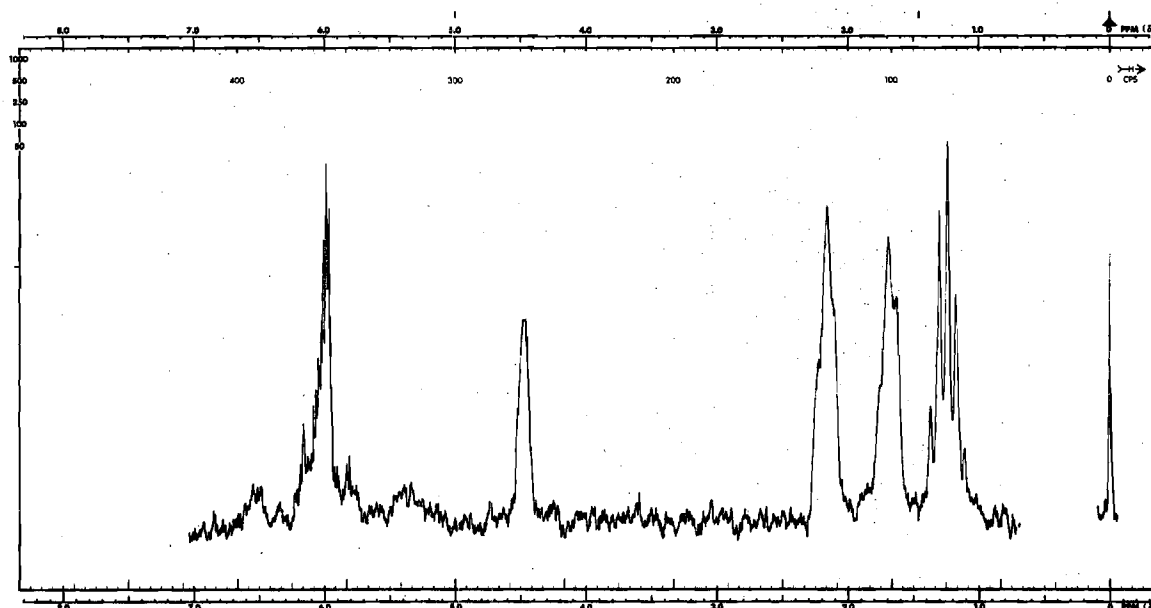


Figure 7. Nuclear Magnetic Resonance Spectrum of  $\Delta^1$ -Pyrroline in Trifluoroacetic Acid Solution.

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