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THE CHEMISTRY OF VIOMYCIN:
THE GUANIDO COMPOUND

A THESIS

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SUMMARY

The principal guanido compound present in the acid hydrolysate of Viomycin has been purified by the use of ion exchange resins and by carbon chromatography. The compound was obtained crystalline from an alcohol-water solution. Elemental analysis of the crystalline compound revealed the empirical formula for the hydrochloride salt to be $C_6H_{10}O_2N_4 \cdot HCl$.

The guanido compound was found to be optically active, exhibiting $[\alpha]_D$ of -78° in water. Potentiometric titrations showed the compound to have pK_a values of 2.8, 5.87, and 13.4 in 66 per cent dimethylformamide and 5.30 and 12.6 in water.

The compound was subjected to acidic and basic hydrolyses in an effort to degrade the guanido compound to simpler compounds which could be more easily identified. Papergrams of the hydrolysis mixtures revealed them to be complex. None of the products was identified. The amount of volatile base released by basic hydrolysis was measured and compared with the behavior of known compounds.

The guanido compound was reduced by one mole of hydrogen under perhydrogenation conditions. After purification,

the only crystalline product obtained was the starting material, recovered in 20 per cent yield.

The data obtained from nuclear magnetic resonance, infrared, and ultraviolet absorption spectroscopy proved to be of exceptional value in the elimination of many structures from consideration.

In the course of this research, 4-aminopyrrolidine-2-carboxylic acid (aminoproline) was synthesized.

I. INTRODUCTION

A. The Chemistry of Viomycin.--Viomycin, a tuberculostatic antibiotic, was isolated simultaneously in 1950 in the laboratories of Chas. Pfizer and Co. and Parke Davis and Co. (1,2) from similar cultures of Streptomyces with red-violet mycelia (Streptomyces puniceus and Streptomyces floridae, respectively). The two isolates were exchanged and were seen to be identical.

Biological studies indicated that Viomycin might be a useful antibiotic, since it was effective in protecting mice against Mycobacterium tuberculosis H37R_v (1). However, subsequent studies on Viomycin with humans in the advanced stages of tuberculosis revealed that kidney toxicity, electrolyte imbalance, vestibular dysfunction, and hypersensitivity resulted from the administration of Viomycin over periods of two to ten weeks (3). Viomycin is still used clinically, however, especially as an antituberculostatic drug in cases in which the tuberculosis microorganism has become resistant to the action of streptomycin.

Viomycin was isolated and purified as the sulfate salt by use of carbon chromatography. Various salts of

Viomycin have been prepared (sulfate, hydrochloride, picrate, and the reineckate) all of which are soluble in water but virtually insoluble in organic solvents. The sulfate melts at 252° with decomposition and has a specific rotation of -39.80° (2).

Viomycin gives positive Sakaguchi, ninhydrin, and biuret tests and negative maltol and Benedict's tests, indicating that it contains guanido and peptide groups, but probably does not contain a carbohydrate component. Viomycin sulfate shows an ultraviolet absorption maximum which is dependent upon the pH of the solution: $\lambda_{\text{max.}}$, 268 m μ , $E_{1\%}^{1\text{cm.}}$ 339 (0.1N HCl); $\lambda_{\text{max.}}$, 268.5 m μ , $E_{1\%}^{1\text{cm.}}$ 334 (pH 7); $\lambda_{\text{max.}}$, 282.5 m μ , $E_{1\%}^{1\text{cm.}}$ 219 (0.1N NaOH) (2).

The free base of Viomycin was prepared by passing Viomycin sulfate over Amberlite IRA-400 ion exchange resin in the hydroxyl phase. The effluent was frozen and lyophilized. The infrared spectrum of the free base was determined in Nujol mull. Absorption maxima were observed at 3.04 μ , 3.66 μ , 5.8 to 6.1 μ with a shoulder at 5.72 μ , and 13.9 μ (4).

Elementary analysis on Viomycin sulfate gave C, 35.89; H, 5.52; N, 21.15; S, 5.79 (4). Van Slyke amino

nitrogen determination indicated 1.65 moles of primary amino nitrogen per mole of Viomycin (5).

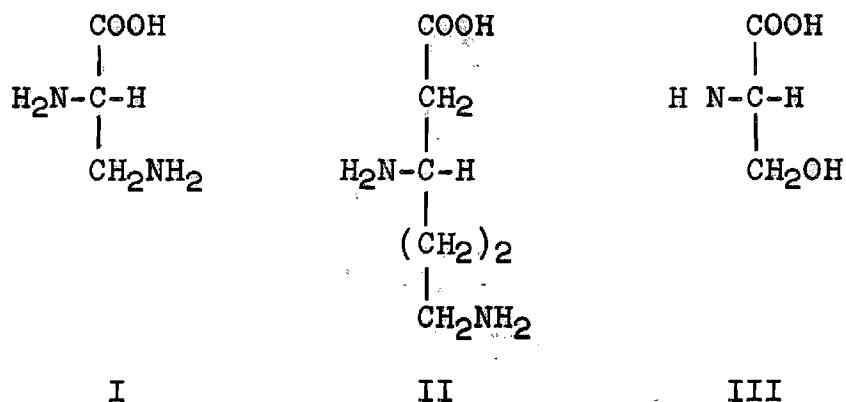
The empirical formula $C_{18}H_{33}N_8O_8$ (molecular weight 500) was reported by Haskell and coworkers (5). The molecular formula $C_{25}H_{40-48}N_{12}O_{11} \cdot \frac{3}{2}H_2SO_4$ was suggested for Vinactin A sulfate, which has been shown to be identical with Viomycin (4). The molecular weight of the free base of the C_{25} compound is 688. Diffusion molecular weight studies indicate a molecular weight of 625 (4), which would seem to indicate that the C_{25} formula is preferable.

Viomycin is a strong base showing pK_a values of 8.2, 10.3, and 12 (4). There is probably no free carboxyl group in Viomycin. The pK_a of 12 is probably due to the guanido group and the pK_a of 10.3 to a primary amino group. The pK_a of 8.25 may be due to an α, β -unsaturated or polar substituted amino compound (4).

Viomycin has isosbestic points at 235 $m\mu$ and 281 $m\mu$, which probably indicates that only one group is dissociating (4).

Viomycin, when hydrolyzed with boiling 6N hydrochloric acid under reflux, yields at least seven compounds: carbon dioxide, ammonia, urea, L- α, β -diaminopropionic acid,

(I), L-lysine, (II), L-serine, (III), and a mixture of



guanido compounds, one of which is present in the hydrolysate in reasonable amounts (5). Hydrolysis in alkaline medium yields, in addition to the compounds observed with acid hydrolysis, a compound which appeared to be alanine by comparison with authentic alanine on paper chromatograms (4).

Reduction of periodic acid by Viomycin sulfate was determined at pH 2.0, 4.55 and 8.38. Viomycin sulfate reduced 1.06 to 1.31 moles of periodic acid in two minutes at pH 2.0 and 4.55. No further reduction was observed in the next 22 hours at 25°. At pH 8.38, 1.18 moles were reduced in two minutes, 1.5 moles in 60 minutes, 2.1 moles in 5 hours and 4.1 moles in 21.5 hours. During the periodate oxidations, no formaldehyde, formic acid or ammonia was produced. However, subsequent experiments revealed that

the first mole of periodate reduced by Viomycin actually represents iodine reduced by Viomycin and not periodate, and also that periodic acid oxidation of Viomycin occurs at a relatively slow rate (4).

In permanganate oxidation experiments Viomycin sulfate was determined to undergo an electron change of 4.9 to 5.2 electrons in neutral media. The reaction was rapid. It was determined that the removal of four electrons instead of five from Viomycin destroyed the ultraviolet chromophore. This agrees with the data from the periodate oxidations, which indicated that the chromophore was destroyed by two moles of periodate (four electrons). However, the removal of only two electrons by iodine destroyed the chromophore. In the permanganate oxidations neither carbon dioxide nor oxalic acid was produced (4).

Attempted hydrogenations with Adam's catalyst at 37° and atmospheric pressure or with palladium on Norite in Adam's apparatus at 42 pounds per square inch and 25° did not alter the shape and position of the ultraviolet peak. Evidently no readily reducible group is involved in the chromophore (4).

A solution of Viomycin was incubated with papain-cysteine for several days. It was shown by means of paper chromatography that papain had no effect on Viomycin (4).

The 2,4-dinitrophenyl derivative of Viomycin was prepared from 2,4-dinitrofluorobenzene in sodium bicarbonate, water, and ethanol solution at room temperature (4). Under these conditions 2,4-dinitrofluorobenzene has been shown to react with primary amino groups, phenolic hydroxyl groups, and one of the imidazole nitrogens of histidine, but not with guanido nitrogens or with the hydroxyl group of serine (6,7). Hydrolysis of the dinitrophenyl derivative of Viomycin with 6N hydrochloric acid for six hours, followed by two-dimensional papergrams indicated the presence of serine and diaminopropionic acid. However, β -lysine was absent. This shows that one of the amino groups of β -lysine is one of the free amino groups of Viomycin (4). The other group exhibiting basic properties in Viomycin has not been determined.

When Viomycin was hydrolyzed for five hours at 95° with 0.1N hydrochloric acid, it was found that urea was produced but apparently no other fragmentation had occurred. The "des-urea-viomycin" was oxidized with neutral permanganate and then hydrolyzed with 1N hydrochloric acid. Sakaguchi

analyses indicated that the amount of guanido group had been decreased to 25 per cent of its original value. The "des-urea" compound was also observed to donate only 4.2 electrons, instead of the five electrons observed for Viomycin itself, with neutral permanganate (4).

Either urea or the guanido group is involved in the chromophore, since on acid hydrolysis the rates of urea production and guanido increase parallel the decrease in the extinction at 268 m μ . Alkaline hydrolyses indicated that it is urea and not the guanido group that is involved (4).

B. The Chemistry of the Guanido Compound.--Viomycin, when hydrolyzed with 6N hydrochloric acid, yields one principle guanido compound and several other guanido compounds in minor amounts as shown by the use of paper chromatography (4).

The Sakaguchi-positive substances were separated from the other products of hydrolysis. The hydrolysate was passed over a Zeo-rex column; urea is not absorbed and comes off first. Ammonia (0.1N) was used to begin development of the amino acids on the column. L-Serine came off first. To separate the guanido compounds from diaminopropionic acid,

Amberlite IR-400 ion exchange resin in the hydroxyl phase was used. Water was used to elute the guanido compound. The diaminopropionic acid was retained on the column and was eluted with an ammonium carbonate solution (4,5).

The Sakaguchi-positive aqueous eluates from the IR-400 column were neutralized and dried in the frozen state. The isolate had a specific rotation in water of -75.3° and in $0.5N$ hydrochloric acid of -9.46° . It did not give a positive Tollen's test. The isolate, with Benedict-Behr reagents, gave no color. When heated under acidic conditions strenuous enough to convert creatine to creatinine, the isolate retained its reactivity with Sakaguchi reagents and still did not give a color with Benedict-Behr reagents, indicating that no cyclization to give a creatinine type of linkage occurred (4).

Several attempts were made to isolate a pure guanido compound. The isolate was chromatographed on acid-washed Solka-floc (cellulose) but no separation was obtained. Chromatography on Amberlite XE-64 carboxylic cation exchange resin in the hydrogen phase was tried but again no purification resulted. Amberlite XE-67 in the hydroxyl phase was tried and some separation was obtained. From 900 mg.

of material 260 mg. of material was isolated in one major fraction (4).

The flavinate and p-hydroxyazobenzene-p'-sulfonate salts were prepared from the major fraction and submitted for analysis. The molecular formula for the free base of the guanido compound was indicated to be $C_6H_{12}N_4O_3$ by analysis of the p-hydroxyazobenzene-p'-sulfonate salt and $C_6H_{16}N_4O_6$ by analysis of the flavianate salt. The optical rotation of the purified guanido compound in water was -63° (4).

The guanido compound was reported to have pK_a values of 1.5, 5.7 and 12.4 (4). The value of 1.5 can probably be attributed to a carboxyl group. The 5.7 value is probably due to a weakly basic amino group while the guanido group is responsible for the pK_a of 12.4. The guanido compound absorbed ultraviolet light, showing a maximum at 216 $m\mu$, ϵ , 220 (4).

It was found that the guanido compound reduced 0.26 mole of periodate in 30 minutes and 1.05 moles in 6 hours at pH 8.4, indicating the probable absence of vicinal hydroxyl or amino groups (4).

The guanido compound was shown to be a mixture, since four spots were obtained on two-dimensional papergrams (4).

After hydrolysis of the guanido compound with hot saturated barium hydroxide under reflux for three hours a negative Sakaguchi test was obtained. Paper chromatograms of this hydrolysate revealed three spots (4).

Guanido compounds less basic than the major one were reported, and attempts to isolate any of them in a pure state were unsuccessful (4).

II. EXPERIMENTAL

A. Apparatus and Techniques

1. Ion Exchange Resins

Ion exchange resins as received from the manufacturer were prepared for use by treatment with $4N$ hydrochloric acid with occasional stirring during an hour, followed by decantation and washing of the resins with water on a large funnel until the pH of the water effluent was 2.0. The resins were then treated with $4N$ sodium hydroxide in the same way, except that the pH of the water effluent was 6.0. This process was repeated three times. The final washing of the ion exchange resins with water was carried out counter-currently in a column. For resins to be used in the hydrogen phase, the final pH of the water wash was 2.5 to 3.0; for resins to be used in the hydroxyl phase, the final pH of the water wash was 7.0 to 8.0.

The preparation of IR-400 ion exchange resin required a slightly different procedure in that the period of stirring was about 12 hours and the resins were stirred continuously.

The ion exchange resins were used in two ways: batchwise and in a column. When the ion exchange resins were used batchwise they were added to the solution in a flask and swirled occasionally during twenty minutes. The progress of the action of the ion exchange resins could be determined by measuring the pH of the solution with p-Hydrion paper. When the ion exchange resins were used in columns, the ion exchange resins were added to columns of suitable diameter so that the height of the resins was as high as practical. The amounts of resins used were approximately five times the amount theoretically needed. The resins in the columns were washed with two column volumes of water before the addition of the sample. After application, the sample was washed through the column by several column volumes of water.

2. Paper Chromatography

a. Apparatus.--The chromatography tanks used were 24 cm. in diameter and 49 cm. in height. The tanks were covered with glass plates sealed by stopcock grease. The solvent systems were added until they were approximately 2 cm. in depth. An all-glass atomizer was used for spraying the paper chromatograms with various reagents. Compressed air was used in spraying the papergrams. The paper used was Whatman No. 1.

b. Solvent Systems.--A phenol-water solvent system was prepared by mixing colorless phenol and water in the proportion 4:1 by volume. The t-butyl alcohol-acetic acid-water solvent system was prepared by mixing t-butyl alcohol, glacial acetic acid and water in the proportions 2:1:1 respectively, by volume. The n-propyl alcohol-acetic acid-water solvent system was prepared by mixing n-propyl alcohol, glacial acetic acid and water in the proportions 10:1:9 respectively, by volume. Throughout this thesis, these solvent systems are referred to as PW, BAW and PAW, respectively.

c. Techniques.--Solutions of the substances were applied to the paper by means of small capillary tubes. The diameters of the applied spots were about 1.0 cm. In general, about 10-20 mcg. of a pure substance was applied or about 40-100 mcg. of a mixture of substances. After the sample had been applied, the papergram was placed in the chromatography tanks and the solvent was allowed to rise to within four cm. from the top of the papergram; this generally required eight to ten hours. The papergram was then removed, allowed to dry, and sprayed with the appropriate reagent.

d. Spray Reagents.--The ninhydrin spray reagent (8,9) was prepared by dissolving 0.2 per cent 1,2,3-triketohydrindene hydrate in 50 per cent aqueous pyridine. When the papergrams were dry, they were sprayed with the reagent and allowed to dry at room temperature for an hour and then heated briefly over a hot plate. Positive results were indicated by the appearance of a typical purple spot, although compounds of certain structural types are known to give red, brown, or yellow colors.

The Weber spray reagent (9) was prepared by mixing equal volumes of 10 per cent sodium hydroxide, 10 per cent sodium nitroprusside, and 10 per cent potassium ferricyanide, and diluting the mixture with three volumes of water. After about twenty minutes the initial dark color of the solution changed to a pale yellow color and the solution was ready for use. The reagent was unstable at room temperature. Mono- and disubstituted guanidines gave orange to blue colors with this reagent, while symmetrically trisubstituted guanidines do not give a color (9). The colors were usually unstable and faded rapidly.

A spray reagent for the detection of urea and ureido compounds (9) was prepared by adding 2.0 g. of p-dimethylamino-benzaldehyde to 100 ml. of 1.2N hydrochloric acid. The

solution was prepared fresh each time it was used. Urea gave bright yellow spots against a white background. The colors did not fade on drying or standing.

An isatin spray reagent (10) was prepared by dissolving isatin (0.4 per cent) in butanol containing four per cent acetic acid. Proline gives a blue color with this reagent. Amino acids give a variety of colors, mostly shades of blue, green and purple.

3. Qualitative Color Tests

For the Sakaguchi test (9), five drops of a cold 0.1 per cent solution of α -naphthol in a 1N sodium hydroxide was added with shaking to a cold solution of the compound to be tested (ca. 2 mg.). To this solution was added five drops of a saturated solution of bromine in 1N sodium hydroxide. The test solutions were kept cold for a half hour to test the stability of the color. Monosubstituted guanidines gave a red color which was stable after a half hour in the cold.

The ninhydrin test was performed by adding two drops of the solution to be tested to 0.5 ml. of water and 0.5 ml. of the ninhydrin reagent (0.2 per cent ninhydrin in

50 per cent pyridine). The solution was heated on a steam bath for up to ten minutes and the purple color obtained was compared with that of a blank.

For the Weber test a drop of the solution to be tested was placed on Whatman No. 1 paper and the paper was sprayed with the Weber spray reagent.

For testing with Nessler's reagent (11) one drop of the solution to be tested was diluted with 0.5 ml. of water and two drops of a solution of 11.5 g. of mercuric iodide and 8.0 g. of potassium iodide in 100 ml. of 3N sodium hydroxide. An orange color was obtained in the presence of ammonium ion, except in concentrated solutions of ammonium ions, in which case a brown precipitate was obtained.

Chloride ions were detected by diluting one drop of the solution to be tested with 0.5 ml. of water and adding one drop of five per cent silver nitrate solution. The presence of chloride ions was detected by the white precipitate which formed immediately.

4. Carbon Chromatography

The carbon columns were prepared by mixing acid-washed Celite 545 and pretreated (12) Darco G-60 in a ratio

of 1:2 by weight. The Darco was prepared by adding 3.0 g. of oleic acid in 400 ml. of absolute alcohol to 100 g. of Darco. The mixture was allowed to stand for an hour with occasional swirling, and was then diluted with ca. 4000 ml. of distilled water. The Darco was allowed to settle and the supernatant liquid was carefully decanted. This process was repeated twice. Fifty grams of acid-washed Celite 545 was added, the mixture swirled, and poured into a column. The Darco-Celite mixture was washed with ten column volumes of distilled water before use.

5. Cellulose Chromatography

Cellulose chromatography columns were prepared by packing thin layers of dry acid-washed cellulose into the tube by means of a cork attached to the end of a thick glass rod. The dry sample to be placed on the column was ground in a mortar with some cellulose. The sample-cellulose mixture was applied on the column and two more portions of cellulose were added to the mortar, ground and added to the column in the same way as before.

6. Miscellaneous

All melting points were determined on the Köfler micro hot stage. All optical rotations were determined with a Bellingham and Stanley Ltd. polarimeter. The D line of sodium was used as the light source. All infrared spectra were determined on the Perkin Elmer Model 137 recording spectrophotometer as a mull in Nujol and in hexachlorobutadiene. The ultraviolet spectra were determined on either the Beckman Model D. K. recording spectrophotometer or the Beckman Model D. U. spectrophotometer. The fraction collector which was used in the course of all column chromatographic separations was made by the Research Specialties Co., Model 1205.

B. Isolation of the Guanido Compound

1. Viomycin Hydrochloride

A solution of 27.5 g. of Viomycin sulfate (Parke-Davis Batch No. 212470 A, assumed molecular weight, 835.8) in ca. 70 ml. of water was treated batchwise with ca. 50 ml. of Amberlite IR-45 (OH⁻) ion exchange resin. The pH rose from 4.5 to 8.5. The supernatant liquid was then passed

through an Amberlite IR-45 (Cl^-) ion exchange resin column containing 340 ml. of resin. The column was washed with 1500 ml. of water. The effluent solution was concentrated by distillation in vacuo to a volume of ca. 25 ml. To this solution was added 360 ml. of redistilled acetone; an oil formed. The liquid was decanted and 50 ml. of acetone was added to the oil. A solid formed which was triturated well and collected by filtration on a sintered glass funnel. The Viomycin hydrochloride (assumed molecular weight, 798.2), dried in vacuo overnight, weighed 27.65 g.

2. Hydrolysis of Viomycin Hydrochloride

A solution of 54.9 g. (68.9 mmoles) of Viomycin hydrochloride in one liter of 6N hydrochloric acid was heated for 6 hrs. on a steam bath. The red solution was concentrated to ca. 100 ml. by distillation in vacuo and treated batchwise with 500 ml. of Amberlite IR-45 (OH^-) ion exchange resin. The pH of the resulting solution was 5.5. The supernatant liquid was passed over an Amberlite IR-45 (Cl^-) ion exchange resin column containing 1500 ml. of resin and washed through the column with 3000 ml. of water. The effluent solution was evaporated to dryness

in vacuo and redissolved in 100 ml. of water. The product was subjected to paper chromatography; the results are seen in Tables 1 and 2.

Table 1.

R _f (BAW)	Ninhydrin	Weber	Urea
0.30	purple	red	yellow
0.53-0.64	red	---	---
0.67	---	---	yellow

Table 2.

R _f (PW)	Ninhydrin	Weber	Urea
0.08	---	red	---
0.11	purple	---	yellow
0.14	brownish	red	---
0.20-0.38	purple	---	---
0.69	---	---	yellow

3. Ion Exchange Separation of Viomycin Hydrolysate

The solution containing the hydrolysis mixture was passed over an Amberlite IR-400 (OH⁻) ion exchange resin column containing 1200 ml. of resin. Urea and basic compounds were eluted by washing the column with 2500 ml. of

water. The materials retained by the ion exchange column were eluted by washing the column with 1N hydrochloric acid until the pH of the effluent dropped from 5.5 to 1.0. The column was then washed with water until a negative ninhydrin test was obtained.

The water eluate was passed over an Amberlite IRC-50 (H^+) ion exchange resin column containing 250 ml. of resin. Urea was washed through the column with 750 ml. of water. This fraction was discarded. In a previous separation, 0.247 g. (4.1 mmoles) of urea was obtained from 5.0 g. (6.26 mmoles) of Viomycin hydrochloride in the same way. Urea was shown to be the only substance present in this fraction by paper chromatography.

The basic compounds retained by the Amberlite IRC-50 (H^+) ion exchange resin were eluted with 400 ml. of 1N hydrochloric acid followed by 250 ml. of water. The solution containing the basic compounds was treated batchwise with 500 ml. of Amberlite IR-45 (OH^-) ion exchange resin and the supernatant solution was passed over an Amberlite IR-45 (Cl^-) ion exchange resin column containing 250 ml. of resin. The basic compounds were washed through this column with 750 ml. of water. This guanido fraction was reduced

in volume by distillation in vacuo and lyophilized; it was found to weigh 8.0044 g. Papergram analysis of this fraction is given in Table 3.

Table 3.

R _f (BAW)	R _f (PW)	Ninhydrin	Weber	Urea
0.31		purple	pink	yellow
0.48		purple	pink	---
	0.05	purple	---	---
	0.21	purple	---	---

The solution containing compounds eluted from the Amberlite IR-400 (OH⁻) ion exchange resin column with hydrochloric acid was treated batchwise with 1500 ml. of Amberlite IR-45 (OH⁻) ion exchange resin. The supernatant solution was then passed over a column containing 1500 ml. of Amberlite IR-45 (Cl⁻) ion exchange resin. The compounds were washed through the column with 3000 ml. of water. This fraction, containing neutral and weakly basic amino acids, was reduced in volume by distillation in vacuo and lyophilized; it was found to weigh 36.965 g. Papergram analysis of this fraction is given in Table 4.

The R_f values of the pure compounds known to be present in Viomycin hydrolysate are given in Table 5.

Table 4.

R _f (BAW)	R _f (PW)	Ninhydrin	Weber	Urea
0.32		purple	---	---
0.45		purple	---	yellow
0.55		purple	---	---
	0.11	purple	---	yellow
	0.33	purple	---	---
	0.51	purple	---	---

Table 5.

Compound	R _f (BAW)	R _f (PW)	R _f (PAW)
The guanido compound	0.30	0.15	0.48
Urea	0.67	0.71	---
<u>L</u> -β-Lysine	0.53	0.33	0.52
<u>L</u> -2,3-Diaminopropionic acid	0.44	0.33	0.44
<u>L</u> -Serine	0.54	0.33	0.68

Two dimensional paper chromatograms were run on the original acid hydrolysate of Viomycin hydrochloride. The results obtained are given in Table 6.

Table 6.

R _f (BAW/PW)	Ninhydrin	Urea
0.20/0.05	grayish purple	---
0.35/0.05	grayish purple	---
0.37/0.11	brownish	yellow
0.37/0.15	pink	---
0.46/0.33	reddish purple	yellow
0.46/0.50-0.76	brownish purple	---
0.39/0.83	brownish purple	---
0.61/0.71	---	yellow

4. Carbon Chromatography of the Guanido Fraction

The guanido fractions from two runs were combined (8.8815 g.) and dissolved in a small amount of water (ca. 20 ml.). This solution was pipetted onto a column 6.9 cm. in diameter containing ca. 1200 g. of 1:2 acid-washed Celite 545:Darco G-60 pretreated with three per cent oleic acid. The packed absorbant was 90 cm. high. The column was eluted first with water (302 fractions of about 20 ml. each) and then with 0.01N hydrochloric acid containing two per cent acetone by volume (329 fractions of about 20 ml. each). The fractions were tested with aqueous silver nitrate, Nessler's, ninhydrin, and Weber reagents. The fractions were combined into larger fractions on the basis of these color tests.

Those combined fractions which were acidic were treated batchwise with Amberlite IR-45 (OH⁻) ion exchange resin until the pH increased to 5.5. The supernatant liquids were passed over IR-45 (Cl⁻) columns. The columns were eluted with two column volumes of water. All of the combined fractions were evaporated in vacuo and lyophilized. Table 7 gives the weights of the combined fractions, the identification of the principle materials, and the optical rotations of the fractions.

Table 7.

Fractions	Wt. of Material	Principle Material	\underline{c}	$[\alpha]_D^{30}$
1-150	0.1247 g.	column throw	---	---
151-162	0.6038 g.	NH ₄ Cl	0.922	+10.87
163-168	1.2327 g.	NH ₄ Cl	0.897	+ 1.12
169-174	0.8273 g.	NH ₄ Cl	0.963	+ 2.09
175-191	0.4629 g.	guanido cpd.	1.176	- 1.72
192-302	3.0358 g.	guanido cpd.	1.085	-51.92
303-452	0.5269 g.	guanido cpd.	1.086	-23.13
453-503	0.1029 g.	guanido cpd.	1.007	- 8.02
504-532	0.0494 g.	guanido cpd.	0.696	-10.20
533-543	0.0628 g.	---	1.000	+ 1.00
544-555	0.2849 g.	---	1.030	---
556-572	0.1332 g.	---	1.085	-18.24
573-597	0.1792 g.	---	1.064	- 1.90
598-631	0.0900 g.	---	0.747	-11.18
	7.7165 Total			

In another run, from 53.0 g. of Viomycin sulfate and Viomycin hydrochloride (1:1) was obtained 16.273 g. of a guanido fraction by the previous hydrolysis and ion exchange resin separation scheme. Paper chromatography revealed that this guanido fraction was a mixture of compounds with R_f values 0.30, 0.26 and 0.17 (BAW). The filtrates from crystallizations of the previous guanido fraction and the fractions from the acid eluate of the first column were combined with this guanido fraction (19.28 g. total) and subjected to carbon chromatography. From the major guanido fraction (8.435 g.) however, only 3.223 g. of crystalline guanido compound could be obtained. The filtrates from the crystallizations of the guanido compound showed ninhydrin-positive spots at R_f 0.18, 0.34, 0.58, and 0.86 (BAW); 0.40, 0.51, and 0.65 (PAW); 0.15, 0.18-0.36, 0.38-0.52, and 0.52-0.88 (PW). All of the spots gave positive Weber tests.

5. Crystallization of the Guanido Compound

To a solution of 298.6 mg. of fraction 192-302 dissolved in one milliliter of water was added 1.1 ml. of redistilled absolute ethanol; the compound crystallized

immediately; 0.9 ml. of water was added and the resulting solution was centrifuged. The supernatant liquid was pipetted into a 15 ml. centrifuge tube. The residue was washed with one milliliter of 50 per cent ethanol and centrifuged. The washing was pipetted in with the original supernatant liquid. To the solution was added 3.3 ml. of absolute ethanol with warming. The compound crystallized rapidly. After cooling, the solid was collected on a small Hirsch funnel and air dried. The crystalline material weighed 0.1428 g.

The remainder of fraction 192-302 was treated in a similar way, yielding 1.0634 g. of crystalline material. The filtrates from the two crystallizations were combined and treated in the same manner yielding an additional 0.5502 g. of material.

The three crystalline fractions (1.7564 g.) were combined and dissolved in 7 ml. of water at 60° in a 40 ml. centrifuge tube. The solution was centrifuged and the supernatant liquid was pipetted into another 40 ml. centrifuge tube. To this tube was added 11 ml. of redistilled absolute ethanol; the material crystallized immediately. The crystalline material was collected on a Hirsch funnel,

washed with redistilled absolute ethanol and air dried. It was found to weigh 1.6425 g. This material was recrystallized in the same way, yielding 1.3240 g. This was dissolved in 4.5 ml. of water at 65° and centrifuged. The supernatant liquid was pipetted into another 40 ml. centrifuge tube and 2.0 ml. of centrifuged, redistilled, absolute ethanol was added. The ethanol was added at 65° and the solution was allowed to cool slowly. The crystalline material was collected on a Hirsch funnel and air dried. It was found to weigh 0.4989 g. (Crop I).

To the filtrate was added 20 ml. of absolute ethanol at 60°. This was allowed to cool to room temperature and was then placed in the refrigerator overnight. The crystalline material was collected by filtration, washed with absolute ethanol, and air dried, yielding 0.4479 g. of material (Crop II).

All of the previous filtrates and washings were combined and reduced in volume to about five milliliters. The material was crystallized by the addition of warm ethanol, followed by cooling. The crystalline material was collected by filtration, washed with ethanol, and air dried, yielding 0.5424 g. of material (Crop III).

The analytical sample (Crop I), on heating showed gradual decomposition, mainly between 200° and 208°.

Papergrams of the analytical sample (Crop I) of the guanido compound showed only one spot for each of the solvent systems with R_f values 0.30 (BAW), 0.15 (PW) and 0.48 (PAW).

Analysis*:	Calc'd.:	C, 35.00; H, 5.36; O, 15.48; N, 27.65; Cl, 17.18; Amino-N, 6.91 (for one group); C-methyl, 7.28 (for one group)
$C_6H_{10}O_2N_4.HCl$ (206.64)	Found:	C, 35.16; H, 5.20; O, 14.20; N, 27.59; Cl, 18.24; Amino-N, 0.15; C-methyl, 1.67

C. Properties of the Guanido Compound

1. Physical Constants

a. Optical Rotations.--The optical rotation of the guanido compound (Crop I) was determined in various solvents. The results are given in Table 8.

*C-methyl analysis by Weiler-Strauss; all others by Huffman Microanalytical Laboratories.

Table 8.

Solvent	\bar{c}	$[\alpha]_D^{30}$
2N hydrochloric acid	2.280	- 21.22
0.1N hydrochloric acid	1.840	- 46.20
water (pH 3.5)	1.780	- 77.77
0.1N sodium hydroxide	2.100	-135.03
0.2N sodium hydroxide	2.165	-145.10
1.0N sodium hydroxide	2.230	-155.76
1.0N sodium hydroxide (after 4 hrs.)	2.230	-153.60
1.0N sodium hydroxide (after 21 hrs.)	2.230	-141.98

b. Potentiometric Titration Data.--Potentiometric titration data (13) indicate pK_a values of 2.8, 5.87, and 13.4 in 66 per cent dimethylformamide and 5.50 and 12.6 in water for the guanido compound.

c. Ultraviolet Spectra.--The ultraviolet spectrum of the guanido compound was determined in aqueous solutions over a wide pH range. In acid or neutral solutions, only end absorption was observed. However, in alkaline solution, a peak appeared at 226 $m\mu$ having an extinction coefficient of 3140. The ultraviolet spectra obtained are shown in Figure 1.

d. Nuclear Magnetic Resonance Data.--The nuclear magnetic resonance spectrum of the guanido compound (14) revealed

three types of protons in deuterium oxide in the ratio 2:2:1. Two protons appeared at +90 c.p.s., two at +7 c.p.s. and one at -29 c.p.s. All of the values given are relative to water as zero at 40 c.p.s. The absorption at -29 c.p.s. was split into at least a triplet and possibly a quadruplet, the absorption at +7 c.p.s. was clearly a singlet, and the absorption at +90 c.p.s. was split into a triplet. The nuclear magnetic resonance spectrum is given as Figure 3.

2. Color Tests

The guanido compound and some known compounds were tested with the Weber reagent. The guanido compound, arginine, creatine, glycoamine, and 1,1-dimethylguanidine sulfate all gave pink colors, while with aminoguanidine sulfate and 1,3-diphenylguanidine purple colors were obtained.

With the Sakaguchi reagent the guanido compound, arginine, and guanidoacetic acid gave red colors which were stable after standing for 30 minutes, while aminoguanidine sulfate, and creatine gave orange colors which were stable for at least 30 minutes. Creatinine and 1,1-dimethylguanidine sulfate gave light purple colors which faded in several minutes to a faint orange color.

1,3-Diphenylguanidine and 1,2,3-triphenylguanidine sulfate gave purple colors with a precipitate. The color produced with 1,2,3-triphenylguanidine sulfate faded rapidly.

The pine splinter test was run by heating a small amount of the compound (ca. 3-5 mg.) and about 20 mg. of zinc dust in a test tube and allowing the vapors to come in contact with a pine splinter which had been soaked for 30 minutes in concentrated hydrochloric acid and allowed to dry in air for 30 minutes. The guanido compound, proline, arginine, ornithine, lysine, hydroxyproline, citrulline, and 2,4-diaminobutyric acid turned the splinter red. Guanidine hydrochloride, guanine, pyrrolidine, 2-aminopyrimidine, and canavanine bleached the splinter from brown to white.

The guanido compound gave a negative test with a one per cent ferric chloride reagent.

3. Basic Hydrolysis

a. Sodium Hydroxide.--A stainless steel test tube was fitted with a three-holed rubber stopper, into which was inserted glass tubes. To one tube was connected a funnel,

through which the base was added. Nitrogen which had been bubbled through concentrated sulfuric acid was admitted through one tube to force any volatile base formed out the other tube through a Kjeldahl bulb into a solution of three grams of boric acid in 75 ml. of water. The solution was back titrated with 0.0393N hydrochloric acid to a screened methyl red end point. The indicator was prepared by dissolving 0.25 g. of methyl red in 100 ml. of ethanol and mixing with a solution of 0.186 g. of methylene blue in 100 ml. of ethanol.

The guanido compound and some known compounds were hydrolyzed with sodium hydroxide at 160° (sand bath). To 37.5 mg. (0.181 mmole) of the guanido compound, 52.4 mg. (0.213 mmole) of aminoguanidine sulfate, 40.0 mg. (0.190 mmole) of arginine hydrochloride, and 18.6 mg. (0.195 mmole) of guanidine hydrochloride were added 12 ml. of aqueous sodium hydroxide solution (saturated at room temperature). The results are given in Table 9.

b. Barium Hydroxide.--To a solution of 36.8 mg. of guanido compound in a 5 ml. pear-shaped flask was added 228.9 mg. of barium hydroxide octahydrate. The solution was heated

Table 9.

Time Hours	Moles of volatile base per mole of compound			
	Guanido Cpd.	Aminoguanidine	Arginine	Guanidine
0:15	---	---	0.145	0.121
0:30	0.315	---	0.466	0.101
0:45	---	---	---	1.491
1:00	0.623	---	0.869	---
1:30	---	0.194	---	---
2:00	0.864	0.416	1.128	2.037
2:30	---	0.879	---	---
3:00	1.118	---	---	---
3:15	---	1.521	---	2.363
4:15	---	1.980	---	---
4:30	---	---	1.397	---
5:15	1.280	2.100	---	---
6:30	---	2.220	---	---
7:00	1.367	---	---	2.616
8:00	---	---	1.635	---
9:00	1.486	---	---	---
9:30	---	2.530	---	---
11:30	---	---	1.801	---
18:00	---	---	---	2.909
20:00	1.736	---	---	---
21:45	---	2.825	---	---
22:00	---	---	2.070	---
31:30	1.823	---	---	---
31:45	---	2.877	---	---
43:30	1.953	---	---	---
46:00	---	2.914*	---	---
46:30	---	---	---	---
47:00	---	3.176	---	---
47:45	---	3.330	2.287	---
49:30	2.387	---	---	---
50:00	---	3.526	---	---
51:45	2.966	---	---	---
53:30	3.439	---	---	---
55:30	3.613	3.824	---	---
57:30	3.689	---	2.349	---
67:00	3.748	3.824	---	---

*At this point the temperature of the sand bath was raised to 310°.

on a steam bath for 24 hours. Aliquots (0.4 ml.) were taken at the end of 1, 2, 3, 4, 6, 12, and 24 hours. Each aliquot was treated with sulfuric acid and centrifuged. Each supernatant liquid was treated batchwise with IR-45 (OH^-) ion exchange resin and then passed over an IR-45 (Cl^-) ion exchange resin column. Each aliquot was evaporated to dryness by distillation in vacuo and redissolved in 0.3 ml. of water. Papergrams revealed only one ninhydrin-positive spot (0.31 (BAW), 0.48 (PAW)) for each of the aliquots. All of the aliquots gave positive ninhydrin tests and yellow colors with the urea spray. Aliquots 1-6 gave a positive Weber test and aliquot 7 was negative. With the Sakaguchi reagent positive tests were obtained for aliquots 1-6 but aliquots 4-6 were faint and aliquot 7 gave a negative test.

Barium hydroxide hydrolysis was carried out on arginine and the guanido compound in an apparatus similar to that used in the sodium hydroxide hydrolyses. A two-necked flask and an addition funnel were used instead of the stainless steel test tube and funnel. A Kjeldahl bulb was not used.

To 38.7 mg. (0.22 mmoles) of arginine hydrochloride (column c of Table 10) and to 38.0 mg. (0.184 mmoles)

(column a of Table 10) and 120.9 mg. (0.585 mmoles) (column b of Table 10) of the guanido compound was added 4.3 ml. (12.9 ml. for the 0.585 mmoles sample of guanido compound) of saturated aqueous barium hydroxide solution. The hydrolysis was carried out on a steam bath. The amount of volatile base formed was determined in the same way as for the sodium hydroxide hydrolysis.

In run b of the guanido compound the amount of carbon dioxide liberated was determined by acidifying the reaction mixture and collecting the liberated carbon dioxide in a solution of barium hydroxide. The dried precipitate weighed 51.5 mg. (0.261 mmoles).

All of the barium hydroxide hydrolysates (142.4 mg.) were combined and subjected to ion exchange resin separation. The combined hydrolysate was passed over a Dowex-2 (OH^-) ion exchange resin column. The basic compounds were eluted with water, and passed directly over an IR-45 (Cl^-) column which was eluted with water. The Dowex-2 (OH^-) column was then eluted with 1N hydrochloric acid. This solution was immediately treated with Dowex-1 (HCO_3^-) ion exchange resin batchwise. The pH rose to 4.5. The Dowex-1 (HCO_3^-) ion exchange resin was eluted with water on a funnel. This solution contained

Table 10.

Time Hours	Moles of volatile base per mole of compound		
	Guanido Cpd. a	Guanido Cpd. b	Arginine c
2:00	0.18	---	0.22
2:45	---	0.121	---
4:00	0.53	---	---
5:30	---	0.319	---
6:00	0.90	---	---
8:00	1.13	---	---
9:00	---	---	0.85
10:00	1.53	---	---
17:00	---	1.67	---
18:00	---	1.73	---
19:00	---	---	1.34
21:00	1.94	---	---
22:30	---	1.97	---
23:00	---	---	1.45
24:00	2.08	---	---
25:00	---	2.07	---
27:00	2.20	---	1.53
29:30	---	2.18	---
31:00	2.32	---	---
36:00	2.47	---	---
41:30	---	2.38	---
42:00	---	---	1.616
45:00	---	2.47	---
48:00	2.58	---	---
50:00	---	2.53	---
65:30	---	2.67	---
77:00	2.75	---	---

neutral compounds. The acidic compounds were eluted from the Dowex-1 (HCO_3^-) resin with 1N hydrochloric acid. The neutral compounds were passed over an IR-45 (Cl^-) ion exchange resin column. The column was eluted with water.

The three fractions were evaporated to dryness by distillation in vacuo; the following weights were obtained: basic fraction 109.6 mg., acidic fraction 41.9 mg., and the neutral fraction 84.4 mg. Papergrams were run on these fractions. The acidic fraction did not give a color with ninhydrin; it was fused and found to be inorganic. Two dimensional papergrams (ninhydrin) were run on the neutral (5 spots) and basic (2 spots) fractions; no Weber-positive spots were obtained. The R_f values obtained were (BAW/PAW) 0.28/0.65, 0.32/0.55, 0.46/0.61, 0.59/0.72, and 0.63/0.72 for the neutral fraction and 0.67-0.38/0.13-0.68 and 0.54/0.74 for the basic fraction. The R_f values for glycine, β -alanine, and α -alanine were 0.46/0.60, 0.66/0.70 and 0.60/0.68 respectively.

4. Acid Hydrolysis

Various amounts of the guanido compound were hydrolysed with 12N hydrochloric acid in a sealed tube in a steam bath for varying amounts of time: 10.5 mg. for 6 days, 4.5 mg. for 4 days, 4.0 mg. for 2 days, and 4.1 mg. for 1 day. Each of the reaction mixtures was evaporated to dryness by distillation in vacuo and treated batchwise with IR-45 (OH^-)

ion exchange resin followed by treatment with IR-45 (Cl^-) ion exchange resin in a column. The resins were washed with water. Papergrams were run on the hydrolysis mixtures. The results are given in Table 11. All of the spots were ninhydrin positive; the Weber positive spots are denoted by (W^+). The Weber spray was not used with the PW solvent system.

Table 11.

	1 day	2 day	4 day	6 day
R_f (BAW)	0.26 (W^+) 0.44 0.52	0.14 0.25 (W^+)	0.26 (W^+) 0.44	0.14 0.29 (W^+) 0.42
R_f (PW)	0.06 0.10 0.17 0.30	0.06 0.10 0.14-0.40 0.43	0.04 0.07 0.10 0.16 0.27	0.07 0.10 0.14 0.19 0.26 0.36
R_f (PAW)	0.51 (W^+) 0.63	0.53 (W^+) 0.60	0.50 (W^+) 0.60	0.54 (W^+) 0.62

To a mixture of 97.0 mg. (0.47 mmoles) of the guanido compound and 20 ml. of absolute methanol was added one drop of concentrated sulfuric acid. The solution was stirred by means of a magnetic stirrer for two days. A white insoluble

solid was separated by filtration and air dried. The solid weighed 46.2 mg. Papergrams of the solid indicated that it was not the starting material: R_f 0.14 (BAW), 0.10 with a head streaked to 0.78 (PW, 0.40 (PAW). The infrared spectra of the solid in Nujol and in hexachlorobutadiene mull showed absorption maxima at 3.22μ (shoulder at 3.08μ), 5.86μ , 5.97μ , 6.33μ , 7.77μ , 8.08μ , 8.59μ , and 12.50μ .

D. Preparation and Properties of the Dihydroguanido Compound

1. Preparation of the Dihydroguanido Compound

The guanido compound (200.6 mg., 0.971 mmoles) was hydrogenated in an acidic medium (80 ml. of 50 per cent acetic acid) at atmospheric pressure and room temperature, using ten per cent platinum on carbon (810.6 mg.) as the catalyst. The guanido compound took up 21.0 ml. (0.94 mmoles) of hydrogen in 166 hours. The catalyst was removed by filtration. The filtrate was concentrated by distillation in vacuo and treated batchwise with IR-45 (OH^-) ion exchange resin. The pH rose to 5.5. The supernatant liquid was passed over an IR-45 (Cl^-) ion exchange resin column containing 30 ml. of resin. The column was washed with 100 ml. of water.

The eluate was evaporated to dryness by distillation in vacuo. After drying in vacuo over calcium chloride, the dihydro compound weighed 0.2347 g. Papergrams of the dihydro compound showed two ninhydrin- and Weber-positive spots: 0.34 and 0.52 (BAW), 0.57 and 0.72 (PAW).

An amount of 1.4872 g. (7.22 mmoles) of the guanido compound was hydrogenated as above, using 6.028 g. of 5 per cent platinum on carbon and 50 ml. of 50 per cent acetic acid. The reaction mixture took up 172.2 ml. of hydrogen (8.0 mmoles) in 28 hrs. The hydrogenation product was worked up in a similar way as the previous hydrogenation product. The amount of hydrogenation product obtained was 1.755 g.

2. Purification of the Dihydroguanido Compound

The dihydro compound from hydrogenation of 200.6 mg. of the guanido compound was applied to Whatman No. 17 chromatography paper (11.9 cm. x 40 cm. 50 mg. per strip). These strips were hung from glass rods with Nichrome wire. The bottoms of the strips rested on the bottom of the chromatography tank, so that the solvent system used, BAW, extended about two centimeters up the strips. The solvent

front was allowed to rise to within two centimeters of the tops of the strips (20 hrs.). The positions of the two spots were determined by spraying thin slices (0.5 cm. wide) with the ninhydrin spray reagent. The spots were cut out and eluted with water. The two fractions were evaporated to dryness in vacuo, and then dried over calcium chloride in vacuo. The fraction with the lower R_f ("bottom" dihydro) weighed 154.3 mg. and the fraction with the higher R_f ("top" dihydro) weighed 62.3 mg. Papergrams of the fractions showed spots with R_f values 0.30 in BAW for the "bottom" dihydro and 0.30 and 0.43 in BAW for the "top" dihydro.

The hydrogenation product obtained from 1.4872 g. of the guanido compound was applied to a cellulose column containing 150 g. of cellulose as described in section A. The column was eluted under nitrogen pressure with the BAW solvent system. Fractions of about 10 ml. volume were collected and tested with the ninhydrin reagent. Positive tests were obtained for fractions 15-60, fractions 31-56 giving the strongest tests. Papergrams revealed one spot for each fraction at R_f 0.29-0.65 (BAW): 0.48 (PAW) and 0.30-0.78 (PW). Fractions 31-56 were combined and evaporated to dryness by distillation in vacuo. The material was then

treated with IR-45 (OH⁻) ion exchange resin batchwise. The supernatant liquid was passed over an IR-45 (Cl⁻) column containing 40 ml. of resin. The column was washed with 100 ml. of water; the resultant solution was evaporated to dryness, then redissolved in two milliliters of water. The solution was pipetted onto a carbon column containing ca. 100 g. of the pretreated Darco-acid washed Celite mixture. The column was eluted with water under nitrogen pressure. Fractions containing ca. 10 ml. were collected automatically. The fractions were tested with aqueous silver nitrate, ninhydrin and the Weber reagent. Papergrams revealed one ninhydrin- and Weber-positive spot with R_F values: 0.32-0.57 (BAW), 0.52 (PAW) and 0.26-0.82 (PW). Fractions 23, 24, 25, 26, 28, and 29 were combined and fractions 22, 30, 31, 32, 33, and 34 were combined, while fraction 27 was kept separate. The combined fractions were concentrated by distillation in vacuo and lyophilized. The major fraction (23-26, 28-29) weighed 1.006 g., fractions 22, 30-34 weighed 0.738 g., and fraction 27 weighed 0.227 g.

3. Barium Hydroxide Hydrolysis

To 4.4 mg. of the "bottom" dihydro compound and 2.9 mg. of the "top" dihydro compound, separated by thick paper

chromatography were added 0.60 ml. and 0.35 ml. of saturated aqueous barium hydroxide respectively in five milliliter pear-shaped flasks. The materials were heated on a steam bath for 54 hrs. At the end of that time they were worked up in a manner similar to that described for barium hydroxide hydrolyses products from the guanido compound. Papergrams were run on the hydrolysis products and the results are given in Table 12.

Table 12.

	R _F (BAW)	R _F (PAW)	R _F (PW)
"bottom" dihydro	0.15 W ⁺ 0.22 0.25 W ⁺ 0.28 W ⁺ 0.62	0.53 W ⁺	0.12 0.22-0.48 W ⁺
"top" dihydro	0.14 W ⁺ 0.22 0.25 W ⁺ 0.28 W ⁺ 0.42	0.00-0.55	0.10 0.22-0.44

The rest of the "bottom" dihydro (149.9 mg.) was dissolved in water and to this solution in a pear-shaped two-necked flask on a steam bath was added 16.94 ml. of saturated barium hydroxide solution. The amount of volatile

base formed was collected and determined in the same way as in the base hydrolyses of the guanido compound. The results are shown in Table 13.

Table 13.

Time hours	moles of volatile base/mole of dihydro cpd.
0	0
3:00	0.57
7:30	0.91
21:00	1.40
25:00	1.53
27:45	1.62
33:45	1.68
45:00	1.74
71:00	1.87

The "bottom" dihydro hydrolysis mixture was worked up in a similar manner as before. Papergrams were run on the reaction product. Ninhydrin-positive spots were obtained with R_f values: 0.26 and 0.38 (BAW), 0.46 and 0.54 (PAW), 0.12 and 0.20 (PW). Both spots were Weber-negative.

4. Crystallization and Properties of the Dihydroguanido Compound

The main fraction from chromatography, 1.006 g., was crystallized from 22.6 ml. of 81 per cent ethanol, yielding

0.2293 g. of crystalline material. The filtrate was concentrated to two milliliters and ethanol was added. From 12 ml. of 81 per cent ethanol was obtained an additional 0.0744 g. of crystalline material. The two crops of crystalline material were combined and recrystallized from 42 ml. of 85 per cent ethanol yielding 187 mg. of crystalline material. The material was recrystallized from 19 ml. of 81 per cent ethanol, yielding 120.3 mg. of crystalline compound. Attempts to obtain additional crystalline material from the filtrates yielded only a green oil.

Papergrams were run on the crystalline material and on the filtrates. The crystalline material showed ninhydrin- and Weber-positive spots with R_f values: 0.34 (BAW), 0.54 (PAW), 0.18 (PW). The filtrates showed ninhydrin- and Weber-positive spots with R_f values 0.36, 0.40-0.62, and 0.80 (BAW), 0.58, 0.68 and 0.75 (PAW), and 0.18, 0.38, and 0.45-0.92 (PW).

Analysis:

$C_6H_{10}O_2N_4.HCl$ Calc'd.: C, 35.00; H, 5.36; N, 27.65; Cl, 17.18
(206.64)

$C_6H_{12}O_2N_4.HCl$ Calc'd.: C, 34.50; H, 6.27; N, 26.89; Cl, 17.03
(208.66)

Found: C, 35.07; H, 5.31; N, 27.49; Cl, 16.97

The optical rotation of the dihydro compound was determined. The specific rotations obtained were -83.18°

(c, 1.6830, in water) and -29.42° (c, 0.9516, in 1.9N hydrochloric acid).

E. Preparation of 4-Aminopyrrolidine-2-carboxylic Acid Hydrochloride (Aminoproline Hydrochloride)

Hydrogenation of 4-nitropyrrole-2-carboxylic acid (15) (2.987 g., 18.49 mmoles) was carried out at atmospheric pressure and room temperature, using a rhodium catalyst (2.03 g. of five per cent rhodium on alumina) in acidic medium (75 ml. of 62.5 per cent acetic acid). The hydrogen uptake was 88.2 mmoles in 42 hrs. The theoretical requirement was 92.45 mmoles of hydrogen. The catalyst was removed by filtration. The filtrate was treated batchwise with IR-45 (OH⁻) ion exchange resin. The pH of the solution rose to 5.5. The supernatant liquid was passed over an IR-45 (Cl⁻) column containing 100 ml. of resin. The column was washed with 200 ml. of water. The solution was evaporated to dryness by distillation in vacuo. Papergrams were run and three ninhydrin-positive spots were observed with R_F values (BAW) 0.28 (yellow), 0.36 (brownish) and 0.60 (light purple); (PAW) 0.58 (tan), 0.67 (pink) and 0.73 (orange); (PW) 0.12-0.27 (brown) and 0.27-0.45 (purple). The p-hydroxyazobenzene-p'-sulfonic acid salt was prepared from

one-half of the material and was recrystallized from hot water. After three recrystallizations 1.341 g. of the crystalline salt was obtained. The hydrochloride was prepared by treating the crystalline p-hydroxyazobenzene-p'-sulfonate salt batchwise with Amberlite IR-45 (OH⁻) ion exchange resin and then passing the supernatant solution over IR-45 (Cl⁻) in a column. The hydrochloride weighed 0.399 g. after drying in vacuo. The aminoproline hydrochloride was crystallized from 65 per cent ethanol. After three crystallizations 0.116 g. of crystalline material (mp. 227-228°) was obtained. The R_F values (ninhydrin) for the pure aminoproline hydrochloride were (BAW) 0.26 (yellowish); (PAW) 0.55 (yellow); (PW) 0.10 (yellow). The infrared spectra are given in Figure 4.

Analysis: Calc'd.: C, 36.01; H, 6.65; N, 16.83; Cl, 21.30

$C_5H_{10}O_2N_2.HCl$ Found: C, 35.91; H, 6.74; N, 15.66, 16.01; Cl, 21.69
 (166.62)

III. DISCUSSION OF RESULTS

The guanido compound has the empirical formula $C_6H_{10}O_2N_4.HCl$ as revealed by elementary analysis. Van Slyke amino nitrogen determination and Kuhn-Roth oxidation of the guanido compound showed no primary amino groups and no C-methyl groups. The guanido compound was a single compound since only one spot was observed on papergrams for each solvent system used.

The guanido compound gave a purple color with the ninhydrin reagent in solution. On paper chromatograms, the color observed was shades of pink-purple, depending on concentration. The intensity of color obtained was somewhat, but not greatly weaker than that ordinarily obtained with α -amino acids. An examination of the literature (8,9) reveals that abnormal colors are frequently obtained with certain kinds of amino acids. The normal requirement for the production of color with the ninhydrin reagent appears simply to be a relatively basic N-H. Tertiary amines do not ordinarily give color. Secondary amines (for example, proline, hydroxyproline, aminoproline, pyrrolidine, piperidine, and N-methylglycine) ordinarily give yellow colors.

It is difficult to attach a particular structural significance to the ninhydrin color given by the guanido compound.

The pink color obtained for the guanido compound with the Weber reagent probably indicates a monosubstituted or N,N-disubstituted guanido group. Pink colors are obtained only with monosubstituted or N,N-disubstituted guanidines (8,9). N,N'-Disubstituted guanidines give colors ranging from blue to purple. The guanido compound gives a relatively stable pink color with the Sakaguchi reagent. Pink or orange colors are obtained with mono- and some N,N-disubstituted guanidines, but negative tests are obtained with most N,N-disubstituted-, N,N'-disubstituted- and trisubstituted guanidines (8,9). Since the guanido compound is stable in strong base for short periods of time, the pink color obtained indicates that the guanido compound probably contains a mono- or N,N-disubstituted guanidine.

The guanido compound did not give any color with aqueous or ammoniacal ferric chloride solution, indicating the probable absence of an enolic grouping.

The pK_a values (13) of 2.8, 5.87 and 13.4 in 66 per cent dimethylformamide and of 5.50 and 12.6 in water are indicative of the presence of a carboxyl group, or other

strongly acidic group, a strongly deactivated amino group, and a guanido group.

The optical activity of the guanido compound showed a marked dependence upon the pH of the medium. By addition of appropriate amounts of acid or base, the optical activity of the compound in a particular condition of ionization could be measured. Assuming the given ionizable groups, the optical rotations of the different ionic species are given in Table 14.

Table 14.

ionic species	$[\alpha]_D$
$-\text{COOH}$, $\begin{array}{c} \\ -\text{N}^+-\text{H} \\ \end{array}$, $\text{NH}_2-\text{C}-\text{N} \begin{array}{l} \diagup \\ \text{NH}_2^+ \end{array}$	-21°
$-\text{CO}_2^-$, $\begin{array}{c} \\ -\text{N}^+-\text{H} \\ \end{array}$, $\text{NH}_2-\text{C}-\text{N} \begin{array}{l} \diagup \\ \text{NH}_2^+ \end{array}$	-78°
$-\text{CO}_2^-$, $-\text{N}-$, $\text{NH}_2-\text{C}-\text{N} \begin{array}{l} \diagup \\ \text{NH}_2^+ \end{array}$	-135°
$-\text{CO}_2^-$, $-\text{N}-$, $\text{NH}_2-\text{C}-\text{N} \begin{array}{l} \diagup \\ \text{NH} \end{array}$	-155°

It is seen that there is a large difference in the optical rotations for the change from the carboxyl group to the carboxylate anion, and also from the ammonium ion to

the amine, while there is only a small difference for the change from the guanidinium ion to the guanido group. It is possible that some stereochemical significance can be attached to these data, but the only conclusion made at this time is that the guanido compound has at least one asymmetric carbon atom.

The ultraviolet absorption spectrum of the guanido compound in acid or neutral solution essentially shows only high end absorption. In alkaline solution, maximum absorption is observed at 226 $m\mu$ with a molar extinction of 3,140. From this bathochromic shift in the ultraviolet spectrum, which changes markedly as the pH of the solutions progresses through the pK_a value for the guanido group, it would appear that the guanido group is involved in the chromophore. These ultraviolet spectra are given in Figure 1.

The differential ultraviolet spectrum of the guanido compound has been examined (13). In this determination, the compound, in solution of a certain pH value, is placed in the sample cell, and the compound, at the same concentration, in solution of a different pH value is placed in the solvent cell. After subtraction of the appropriate

blank spectrum, the resulting differential spectrum is that which is due to the change in an ionizable group because of the different pH values used. For example, if a group has a pK_a value of 5, the differential spectrum using solutions of pH 3 and 7 would be due to the difference in the ionized group and the free group.

When the differential ultraviolet spectrum of the guanido compound was determined using pH 9.80 for the sample cell and pH 3.82 for the solvent cell, it was found that the weakly basic group exhibited $\lambda_{\text{max.}}$, 212 $m\mu$, ϵ , 2,530. In a determination using pH 13.1 for the sample cell and pH 9 for the solvent cell, it was found that the guanido group exhibited $\lambda_{\text{max.}}$, 222 $m\mu$, ϵ , 1,690. The absorption due to the weakly basic amino group appears to be typical of tertiary amines (N-methylpyrrolidine, $\lambda_{\text{max.}}$, 214 $m\mu$, ϵ , 2,300, N-methylpiperdiene $\lambda_{\text{max.}}$, 213 $m\mu$, ϵ , 1,600, 1-azabicyclo[3.3.0]decane, $\lambda_{\text{max.}}$, 215 $m\mu$, ϵ , 3,100). Primary and secondary amines are transparent when subjected to this kind of determination. It is difficult to reconcile the presence of a tertiary amino group present in the guanido compound because of the pK_a values for Viomycin itself. If the guanido compound is present per se, Viomycin

should show a weakly basic group as in the guanido compound. This is not the case.

Arginine shows no differential ultraviolet absorption spectrum using pH values of 10 versus 4, or 13 versus 10. The total ultraviolet spectrum of arginine shows only weak end absorption at any pH value. This would again seem to implicate the guanido group in the guanido compound in some chromophoric system.

The infrared absorption spectrum (Figure 2) of the guanido compound, as determined in nujol and hexachlorobutadiene mulls shows absorption maxima at 2.98μ , 3.18μ , 3.50μ , 3.84μ , 5.91μ , 6.06μ , 6.20μ , 6.33μ , 6.50μ , 6.87μ , 7.10μ , 7.37μ , 7.48μ , 7.84μ , 8.10μ , 8.70μ , 11.16μ , and 12.32μ . The peaks at 2.98μ and 3.18μ appear to be guanidine N-H stretching vibrations. Ordinary amino acids and alcohols do not exhibit sharp absorption bands in this region, but all guanidine derivatives containing N-H show absorption bands in this region. The absorption at 3.50μ is due to C-H stretching vibrations.

The region 5.9μ to 6.5μ contains five relatively strong peaks. In this region, there would be expected to be bands due to C=C stretching, C=N stretching, N-H

deformation, and carboxylate anion vibrations. Mono-substituted- or N,N-disubstituted guanidines exhibit two bands in the 5.90μ to 6.15μ region, usually separated by about 0.10μ (16). N,N'-Disubstituted guanidines exhibit two bands in the 5.93μ to 6.27μ region, usually separated by about 0.30μ (16). The bands present at 5.91μ and 6.06μ in the guanido compound can best be assigned as being due to a monosubstituted- or N,N-disubstituted guanidine. The carboxylate anion absorption of α -amino acids usually appears at $6.29\mu \pm 0.02\mu$, that due to N-substituted α -amino acids at $6.20\mu \pm 0.04\mu$, and that due to β - or δ -amino acids at about 6.38μ (17,18). The absorption present at 6.20μ or 6.33μ could be assigned to the carboxylate anion in the guanido compound. The amino acid I band (18) (N-H deformation) could then be assigned to the 6.33μ or 6.20μ band. The absorption present at 6.59μ could be the amino acid II band (18).

Of the remaining absorption bands present in the guanido compound, the only one which can be assigned with any certainty is that at 6.87μ , undoubtedly due to $-\text{CH}_2-$ deformation vibration. The absorption bands present at 11.16μ and 12.32μ and 12.32μ could possibly be due to

C-H out-of-plane deformation vibrations of an unsymmetrically disubstituted- or trisubstituted olefin, respectively.

The nuclear magnetic resonance spectrum of the guanido compound was determined (14) in deuterium oxide solution and showed absorption maxima at $+90$ c.p.s., $+7$ c.p.s., and -29 c.p.s. (values relative to water as zero at 40 c.p.s.). Two protons were shown to be present at the $+90$ c.p.s. peak, which was split into a triplet, two protons were shown to be present at the $+7$ c.p.s. peak, which was clearly a singlet, and one proton was present at the -29 c.p.s. peak, which was split into a triplet and possibly a quadruplet. The nuclear magnetic resonance spectrum is shown in Figure 3.

The fact that the peak at $+7$ c.p.s. is a singlet indicates that the absorption is due to a methylene group not attached to a carbon atom bearing a proton. The fact that the other two peaks are both split indicates that each carbon is attached to a carbon bearing a proton, and therefore they must be joined.

The $+90$, $+7$, and -29 c.p.s. values for the absorption peaks correspond to chemical shift values (δ) of $+2.25$, $+0.18$, and -0.73 p.p.m., respectively.* An

*The chemical shift values, in parts per million,

examination of the literature values (19) for 16 amino acids of various types reveals the following ranges for the particular kinds of protons: the α -C-H of an α -amino acid, +0.59 to +1.58 (average, +1.10), -CH₂- flanked by two carbon atoms, +3.08 to +3.74 (average, +3.37), C-CH₃, +3.57 to +3.88 (average, +3.74), and -CH₂- adjacent to nitrogen, +1.43 to +2.40 (average, +1.81).

The nuclear magnetic resonance spectrum of the guanido compound clearly indicates that there are five non-exchangeable protons present, and their relative environment. A comparison of the chemical shift values of the guanido compound with ordinary amino acids does not reveal any marked similarity.

An examination of the literature (20) reveals that for ordinary kinds of protons attached to carbon, the only ones which display negative chemical shift values are those which are attached to olefinic carbon atoms. It is significant also that the value for a -CH₂- group flanked only by -CH₂- groups (+3.2 to +3.9 p.p.m.) is markedly changed for a -CH₂- group flanked by one -CH- group and by one -O-

were calculated from the field strength values, in cycles per second, by dividing the observed field strength values by 40, the field strength, in megacycles, used for the determination.

group (+0.9 to +1.8 p.p.m.) (20). For example, the following compounds show, respectively, the following chemical shift values: cyclohexane, -3.9; tetrahydropyran, +1.5 and +3.5; dioxane, +1.5; trioxane, -0.2.

It is not possible at the present time to assign discrete structural units to the nuclear magnetic resonance absorption maxima of the guanido compound. The $-\text{CH}_2-$ group with a δ value of +2.25 appears to be adjacent to an oxygen or nitrogen atom; the $-\text{CH}_2-$ group with a δ value of +0.18 appears to be adjacent to two oxygen or nitrogen atoms; the $-\text{C}-\text{H}$ group with a δ value of -0.73 appears to be attached to an olefinic bond ($\text{CH}_2=$, average δ value, -0.45, and $-\text{C}=\text{C}-\text{H}$, average δ value, -0.67).

In addition to the normal precaution in the interpretation of a nuclear magnetic resonance spectrum of considering the particular environment of a proton, an additional precaution due to the concentration of the compound in the particular determination must be exercised. It has been shown that the chemical shift values change toward zero with dilution (21).

The guanido compound takes up only one mole of hydrogen under perhydrogenation conditions. Assuming there to

be present in the guanido compound a carboxyl group and a guanido group, the empirical formula allows two more double bonds and/or rings. Since one mole of hydrogen was taken up there must be present, in the guanido compound, one double bond and one ring.

Barium hydroxide hydrolysis of the guanido compound released 2.71 moles of volatile base and 0.45 moles of carbon dioxide. Arginine under the same conditions released 1.62 moles of ammonia. This must mean that the guanido group is attached in the guanido compound in such a way as to make a third nitrogen liable to basic hydrolysis. It was anticipated that this relatively mild degradation reaction could lead to a single compound, or a simple mixture of compounds, which would be more easily identified. However, a complex reaction mixture resulted. Papergrams revealed the presence of eight ninhydrin-positive basic and neutral substances.

Sodium hydroxide hydrolysis of the guanido compound at 160° released 1.95 moles of volatile base. The sodium hydroxide hydrolysis of arginine closely paralleled that of the guanido compound, giving 2.23 moles of volatile base in a comparable length of time. Arginine was allowed to react longer under these conditions and released a total

of 2.35 moles of volatile base. Guanidine was hydrolyzed relatively rapidly, giving 2.91 moles of ammonia. Aminoguanidine released 2.91 moles of volatile base in the same amount of time that the guanido compound released 1.95 moles. At this point the temperature was raised to 310°. The guanido compound released a total of 3.75 moles and aminoguanidine 3.82 moles of volatile base.

The guanido compound is hydrolyzed slowly with concentrated hydrochloric acid. After six days at 100° six spots were observed on papergrams. The fact that the guanido compound is destroyed by acid explains the low yield of guanido compound obtained from the hydrolysis of Viomycin. Hydrolysis of 55 g. of Viomycin, assuming the molecular weight of the hydrochloride salt to be 797, should yield 14.2 g. of the guanido compound. Actually only 1.489 g. (about 10 per cent) was obtained. Other guanido compounds are formed during the hydrolysis of Viomycin as evidenced by paper chromatography; none has been isolated in pure form.

The preparation of the methyl ester of the guanido compound was attempted. The product obtained from the reaction mixture exhibited different R_f values than the

guanido compound on papergrams. The infrared spectrum of the product was markedly different than that of the guanido compound, showing absorption maxima at 3.22μ (shoulder at 3.08μ), 5.86μ , 5.97μ , 6.33μ , 7.77μ , 8.08μ , 8.59μ , and 12.50μ . The preparation was not further characterized.

Isolation of the hydrogenation product was attempted. On purification of the reaction product about 20 per cent of the starting guanido compound was obtained as the only crystalline product. The remainder of the reaction mixture was revealed to be a mixture of compounds. Barium hydroxide hydrolysis of a crude product from another hydrogenation released only 1.87 moles of volatile base. This would seem to indicate that hydrogenation had stabilized the third nitrogen which was released on barium hydroxide hydrolysis of the guanido compound. However, no real significance can be attached to this result since a pure dihydroguanido compound was not obtained.

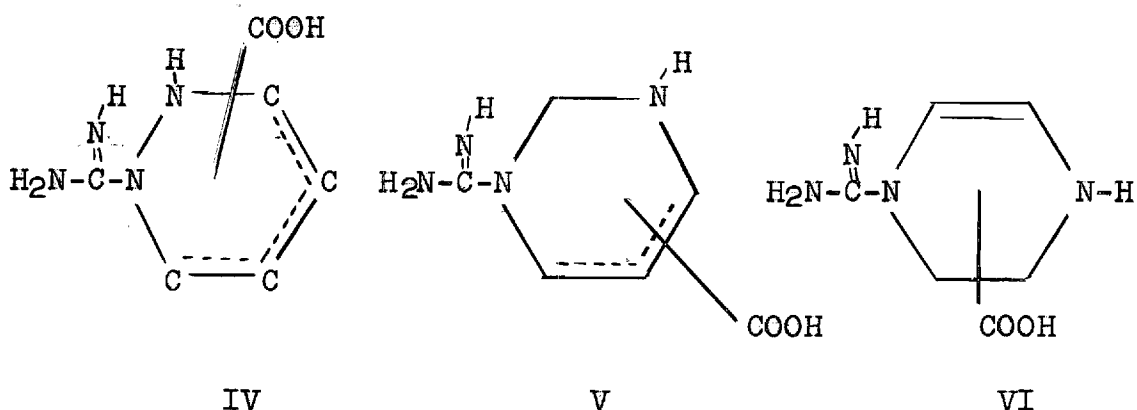
In attempting to derive a possible structure for the guanido compound, α,β -unsaturated carboxylic acids can be excluded (22) on the basis of the ultraviolet absorption spectrum.

Ethyleneimine and propyleneimine structures may be

excluded from consideration on the basis of the fact that these ring systems would not be stable under the hydrolysis conditions by which the guanido compound is obtained (23). Structures containing three and four membered rings can be excluded as possible structures by the nuclear magnetic resonance data. The three membered ring compounds are excluded on the basis of the absence of absorption maxima due to cyclopropane $-CH_2-$. Four membered rings are excluded by virtue of the fact that a structure with five C-H bonds in a ratio of 2:2:1 and not containing any C- CH_3 groups or primary amino groups cannot be drawn.

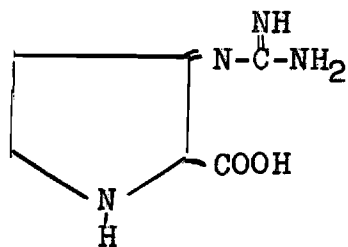
Seven membered rings and larger can be eliminated since they would all require an N,N'-disubstituted guanidine.

Six membered rings require that one of the nitrogens of the guanido group be in the ring. This would give rise to three possible types of structures:

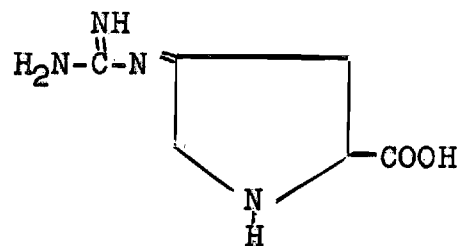


All these structures are excludable on the basis that (1) the guanido compound does not contain an α,β -unsaturated carboxylic acid grouping, (2) the nuclear magnetic resonance data require the groups $-\text{CH}_2-$ and $-\text{CH}_2-\overset{|}{\underset{|}{\text{C}}}-\text{H}$, and (3) the atom in the guanido compound which gives rise to optical activity must be $-\text{CH}_2-\overset{|}{\underset{|}{\text{C}}}-\text{H}$. The tetrahydropyrimidine (V) should react analogously to the hexahydropyrimidines which are reported to be unstable towards both acid and base hydrolysis, readily undergoing ring opening (24). Since the guanido compound was obtained from acid hydrolysis of Viomycin, the tetrahydropyrimidines (V) can probably be eliminated from consideration. Tetrahydropyrazines (VI) seem to be unstable, readily undergoing air oxidation (24).

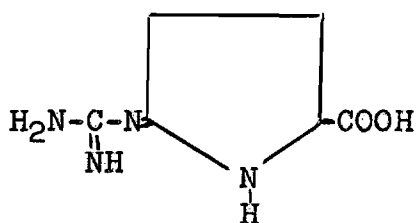
Other structures containing five-membered rings which must be considered are VII-XII. Five membered rings containing $\text{C}=\text{C}$ were excluded since only four C-H would appear in the nuclear magnetic resonance spectrum. Solely on the basis of the nuclear magnetic resonance spectrum (assuming the environment for the five protons to be $-\text{CH}_2-$ and $-\text{CH}_2-\overset{\text{H}}{\underset{|}{\text{C}}}-$), structures VII, IX, X, and XII may be excluded. Chemical evidence against compound IX being the structure of the guanido compound was furnished by barium hydroxide



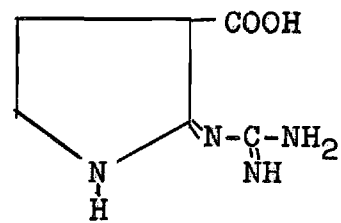
VII



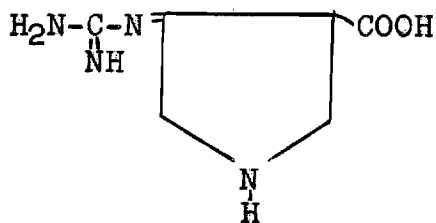
VIII



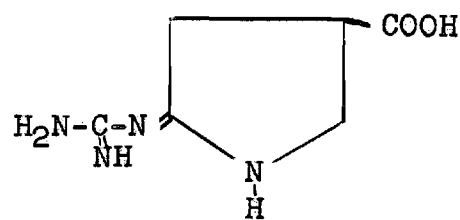
IX



X



XI



XII

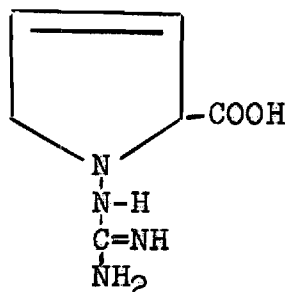
hydrolysis. Barium hydroxide hydrolysis of compound IX would be expected to yield glutamic acid ($\text{HOOC-CH}_2\text{-CH}_2\text{-NH}_2$ CH-COOH). None was obtained. Compound VIII might appear to satisfy the results obtained on barium hydroxide hydrolysis of the guanido compound. Barium hydroxide hydrolysis of the dihydroguanido compound released about two moles of volatile base. If, then, compound VIII were

the correct structure, the product obtained should be 4-aminopyrrolidine-2-carboxylic acid (aminoproline). Papergrams of the barium hydroxide hydrolysis mixture of the dihydro-guanido compound compared with authentic aminoproline revealed that there was no aminoproline in the hydrolysis mixture. How much significance should be placed on this evidence is not known since only the guanido compound was obtained crystalline from the hydrogenation reaction mixture.

It is doubtful that compounds VII-XII would satisfy the observed pK_a values for the guanido compound. For example, the pK_a value of the amine in proline is 10.6 and in hydroxyproline, 9.73. In addition, since all of the compounds studied containing a secondary amino group gave a yellow color with the ninhydrin reagent, it is improbable that one is present in the guanido compound. The nature of the group responsible for the ninhydrin color with the guanido compound remains obscure. All classes of amines are excluded: primary because of the negative Van Slyke amino-nitrogen determination, secondary because of the color which is obtained, and tertiary because such a group (with pK_a 5-6) should be present in Viomycin itself.

A structure suggested on the basis of pK_a values and

ultraviolet absorption spectral data (13) is XIII. This



XIII

structure is excluded by the nuclear magnetic resonance spectral data. In addition, Viomycin has pK_a values of 8.2, 10.3 and 12.0. It shows no pK_a value near 5.50. If structure XIII were correct it would seem probable that attachment of the remaining portion of the Viomycin molecule through the carboxyl group could not increase the pK_a value of the hetero nitrogen to 8.2, which would be necessary since the guanido group is monosubstituted in Viomycin.

All of the structures considered so far contain carboxyl groups. The pK_a (2.8) for the acidic group present in the guanido compound indicates that it is a more strongly acidic group than the carboxyl groups present in ordinary amino acids (lysine, pK_a 3.7, β -lysine, pK_a 5.1). However, a carboxyl group has not been proved to be present by

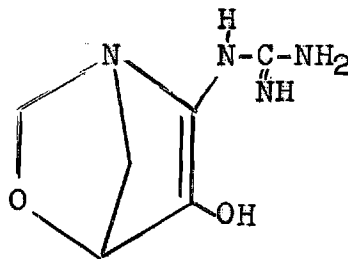
chemical means.

If there were one acidic hydroxyl group present, then the other oxygen could be ethereal. Assuming the presence of one guanido double bond and one reducible double bond, there remains possible two rings, or, less likely, and/or non-reducible double bonds. Assuming the number and environment of carbon-hydrogen bonds as determined by nuclear magnetic resonance to be correct (which also serves to designate the asymmetric carbon atom), there remain to be assigned only four hydrogen atoms. If a tertiary amine group is present, these could be accounted for by a mono-substituted guanidine, whereas if a secondary amine group is present, these could be accounted for by an N,N-disubstituted guanidine. The differential ultraviolet spectral data for a tertiary amine is reasonably convincing. If this is indeed present, one would be nearly forced to the conclusion that the principal guanido compound obtained does not occur per se in the Viomycin molecule.

Employing the above assumptions, it appears that only one structure can be written which is consistent with the nuclear magnetic resonance and ultraviolet absorption data obtained: the bicyclic tetrahydrooxazole derivative, XIV,

1-aza-5-oxa-2-guanido-3-hydroxybicyclo[2.2.1]-hept-2-ene.

However, compound XIV probably does not have the pK_a values observed for the guanido compound.



XIV

Since all of the possible structures for the guanido compound have been eliminated, it is mandatory that one or more of the criteria used in eliminating possible structures is fallacious and must be reinvestigated.

Concentration: 0.408
mmoles per liter

Solvent:

0.05 N HCl -----
0.005 N NaOH -----
0.05 N NaOH -----
0.5 N NaOH
2.5 N NaOH —————

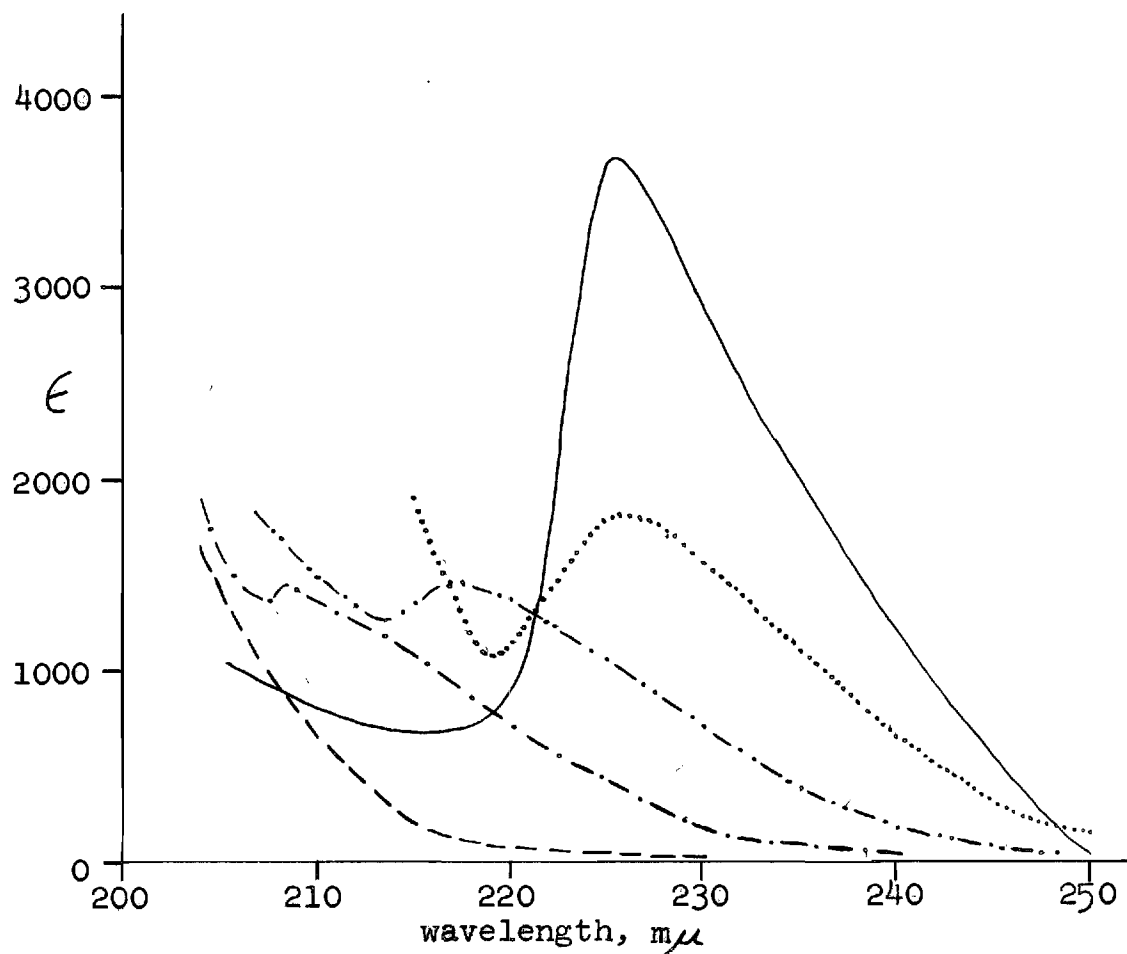


Figure 1. Ultraviolet Spectra of the Guanido Compound

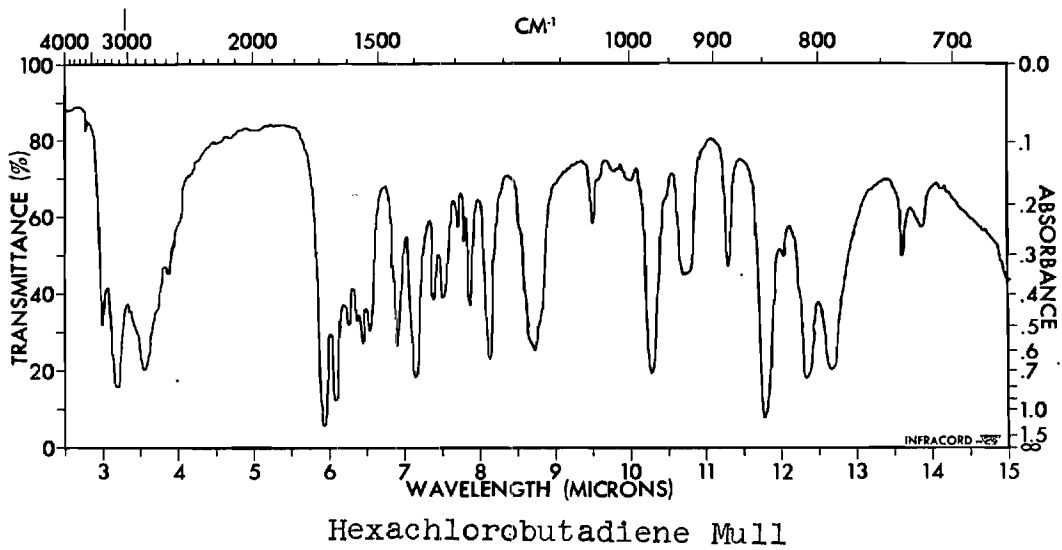
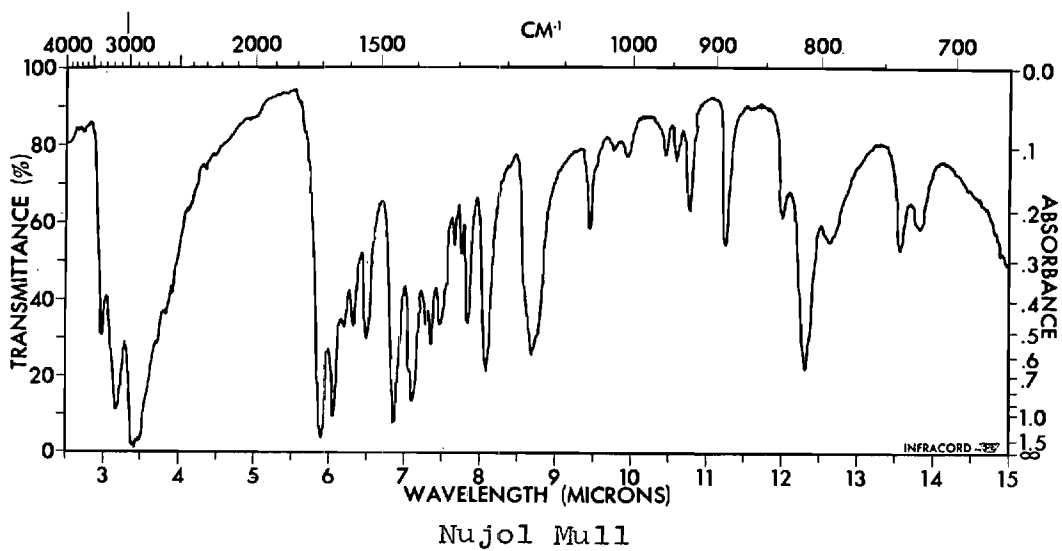


Figure 2. Infrared Spectra of the Guanido Compound

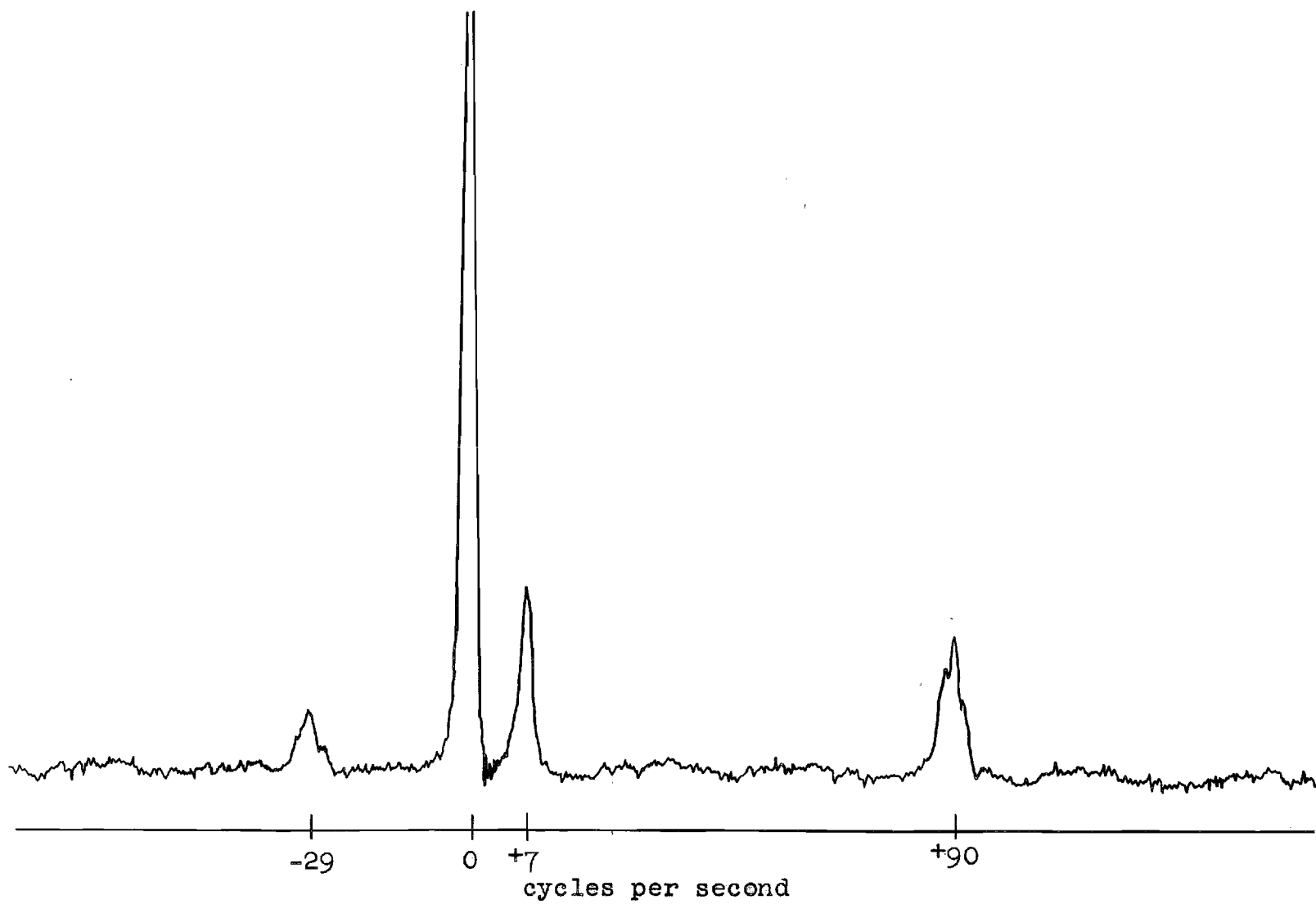


Figure 3. Nuclear Magnetic Resonance Spectrum of the Guanido Compound

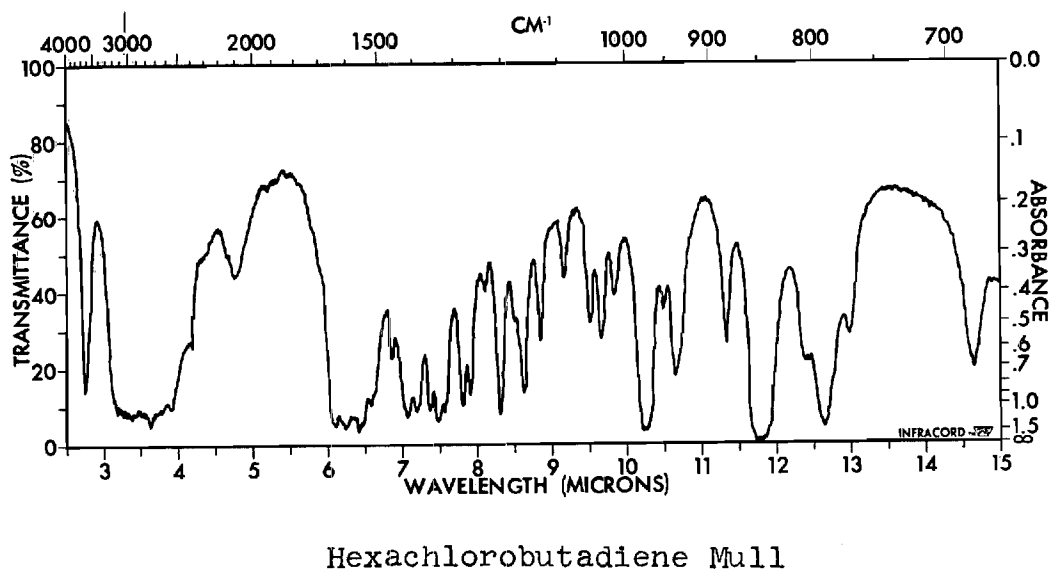
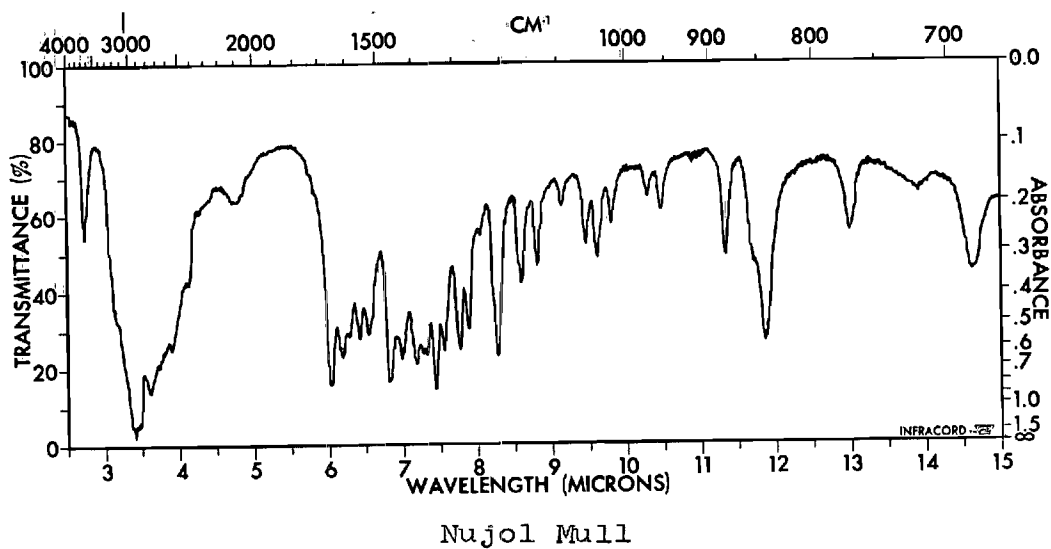


Figure 4. Infrared Spectra of 4-Aminopyrrolidine-2-carboxylic Acid Hydrochloride (Aminoproline Hydrochloride)

Bibliography*

1. A. C. Finlay, G. L. Hobby, F. Hochstein, T. M. Lees, T. F. Lenert, J. A. Means, S. Y. P'Am, P. P. Regna, J. B. Routien, B. A. Sobin, K. B. Tate, and J. H. Kane, Am. Rev. Tuberc., 63, 1 (1951).
2. Q. R. Bartz, J. Ehrlich, J. D. Mold, M. A. Penner, and R. M. Smith, Am. Rev. Tuberc., 63, 4 (1951).
3. C. A. Werner, R. Tompsett, C. Muschenheim, and W. McDermott, Am. Rev. Tuberc., 63, 49 (1951).
4. L. H. Mason, Unpublished Ph. D. Thesis, University of Illinois, 1953.
5. T. H. Haskell, S. A. Fusari, R. P. Frohardt, and Q. R. Bartz, J. Am. Chem. Soc., 74, 599 (1952).
6. F. Sanger, Biochem. J., 39, 507 (1945).
7. S. Blackburn, Biochem. J., 45, 579 (1949).
8. R. J. Block, E. L. Durrum, and G. Zweig, Paper Chromatography and Paper Electrophoresis, Academic Press, Inc., New York (1955).
9. University of Texas Publication No. 5109, University of Texas, Austin, Texas (1951).
10. A. Saifer and I. Oreskes, Anal. Chem., 28, 501 (1956).
11. P. B. Hawk, B. L. Oser and W. H. Summerson, Practical Physiological Chemistry, The Blakiston Company, Toronto (1947).
12. R. L. M. Synge and A. Tiselius, Acta. Chem. Scand., 3, 231 (1949).

*For full titles of journals abbreviated here, consult Chemical Abstracts, 50, 1 (1956).

13. Private communication from Dr. Harold Boaz, Eli Lilly and Company, Indianapolis, Indiana, to Dr. John R. Dyer.
14. Private communication from Dr. K. L. Rinehart, University of Illinois, to Dr. John R. Dyer.
15. W. J. Hale and W. V. Hoyt, J. Am. Chem. Soc., 37, 2538 (1915).
16. T. Goto, K. Nakanishi, and M. Ohashi, Bull. Chem. Soc. Japan, 36, 723 (1957).
17. H. M. Randall, R. G. Fowler, N. Fuson and J. R. Dangle, Infrared Determination of Organic Structures, D. Van Nostrand Company, Inc., New York (1949).
18. L. J. Bellamy, The Infrared Spectra of Complex Molecules, John Wiley and Sons, Inc., New York (1958).
19. O. Jardetzky and C. D. Jardetzky, J. Biol. Chem., 233, 383 (1958).
20. J. E. Wertz, Chem. Rev., 55, 830 (1955).
21. J. D. Roberts, Nuclear Magnetic Resonance, McGraw Hill Book Company, Inc., New York (1959).
22. H. E. Ungnade and I. Ortega, J. Am. Chem. Soc., 73, 1564 (1951).
23. R. C. Elderfield, Heterocyclic Compounds, Vol. 1, John Wiley and Sons, Inc., New York (1950).
24. R. C. Elderfield, Heterocyclic Compounds, Vol. 6, John Wiley and Sons, Inc., New York (1950).