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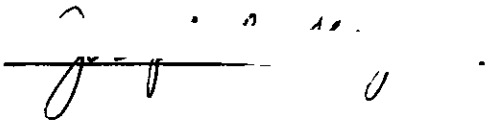

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VIOMYCIN: BIOSYNTHETIC STUDIES AND THE CRYSTAL STRUCTURE OF VIOMYCIDINE

A THESIS

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The Faculty of the Graduate Division

by

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VIOMYCIN: BIOSYNTHETIC STUDIES AND

THE CRYSTAL STRUCTURE OF VIOMYCIDINE

Approved: [signature]

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SUMMARY

Viomycin yields upon complete acid hydrolysis L-serine, L-α,β-diaminopropionic acid, L-β-lysine (L-3,6-diaminocaproic acid), urea, carbon dixoide, ammonia, and a strongly basic amino acid named viomycidine. The purpose of this research was to investigate the biosynthesis of viomycin and in particular to determine the biological precursors of the diaminopropionic acid, β-lysine, and viomycidine residues in viomycin.

Samples of radioactively labelled viomycin were obtained by adding ^{14}C-labelled compounds suspected of being precursors of one or more of the amino acid residues in viomycin to the growth medium of Streptomyces griseus var. purpurea. These samples of viomycin were then hydrolyzed and the hydrolysis components separated by ion-exchange chromatography. The activities of the hydrolysis components were then determined. Chemical degradations were carried out on certain of the hydrolysis components that contained significant activity in order to determine the complete labelling patterns of those components.

Glucose-U-^{14}C, DL-serine-3-^{14}C, DL-serine-1-^{14}C, DL-lysine-2-^{14}C, DL-glutamic-3,4-^{14}C acid, DL-glutamic-5-^{14}C acid, DL-arginine-5-^{14}C, and DL-aspartic-4-^{14}C acid were investigated as possible precursors. A very close similarity between the labelling patterns of the DL-serine-3-^{14}C and DL-serine-1-^{14}C used as precursors and the serine and diaminopropionic acid samples isolated from the hydrolysates

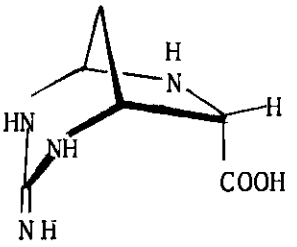
of the corresponding two samples of viomycin was established. These data demonstrated that the complete three carbon unit of serine is the biological of precursor of the diaminopropionic acid residue in viomycin. When DL-lysine-2-^{14}C was used as precursor, the β-lysine isolated from the hydrolysate was significantly active and the activity was predominantly at the C-2 position. It was concluded that lysine is the precursor of the β-lysine residue in viomycin. The fact that significant activity was also found in the serine and diaminopropionic acid isolated from the hydrolysate of this sample of viomycin demonstrated the existence of a new metabolic pathway between lysine and serine. The use of DL-arginine-5-^{14}C as precursor resulted in a relatively high level of incorporation of activity into the viomycidine isolated from the viomycin hydrolysate. It was concluded that it is highly likely that arginine is the precursor of the viomycidine residue in viomycin.

The use of glucose-U-^{14}C as precursor resulted in the incorporation of significant activity in each of the hydrolysis components of viomycin that were isolated. The use of glutamic-3,4-^{14}C acid and glutamic-5-^{14}C acid as precursor resulted in a very low incorporation of activity in the viomycin produced. None of the hydrolysis components were significantly active. The use of aspartic-4-^{14}C acid as precursor also resulted in a very low incorporation of activity in the viomycin produced. The activity of the β-lysine was significantly greater than any of the other hydrolysis components. This incorporation of aspartic acid into β-lysine is consistent with the diaminopimelic acid pathway and provided a reasonable explanation of the fact

that β-lysine from viomycin having serine-3-^{14}C as precursor was significantly active but β-lysine from viomycin having serine-1-^{14}C as precursor had a very low level of activity.

At the beginning of this research the proposed structure for viomycidine was 2-guanidino-Δ^1-pyrroline-5-carboxylic acid. A synthesis of this structure was deemed desirable both to confirm this structure and to provide a synthetic source of viomycidine for use as cold carrier in degradations of labelled viomycidine. L-2-Pyrrolidone-5-carboxylic acid was obtained by heating L-glumatic acid. The O-ethyl imino ether of the methyl ester of L-2-pyrrolidone-5-carboxylic acid was prepared by reaction with triethyloxonium fluoborate. Reaction of the O-ethyl imino ether with a saturated solution of ammonia in ethanol yielded 2-amino-Δ^1-pyrroline-5-carboxamide. All attempts to replace the amino group with a guanidino group failed.

If 2-guanidino-Δ^1-pyrroline-5-carboxylic acid were the correct structure for viomycidine, then the nuclear magnetic resonance spectrum of viomycidine should be quite similar to that of 2-amino-Δ^1-pyrroline-5-carboxamide. When it was found that the two spectra were radically different, the suggested structure for viomycidine seemed to be in question. An X-ray diffraction structure determination was made on viomycidine hydrobromide. The crystal structure of viomycidine was thus shown to be 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid.



Viomycidine

CHAPTER I

INTRODUCTION

Viomycin, a broad spectrum antibiotic, was isolated in 1950 simultaneously by Charles Pfizer and Company *(1)* and Parke, Davis and Company *(2)* from cultures of Streptomyces puniceus and Streptomyces floridae. Early studies indicated that viomycin was particularly effective against Mycobacterium tuberculosis *(1)*, but further studies indicated that the use of viomycin in humans resulted in kidney damage, vestibular dysfunction, electrolyte imbalance, and hypersensitivity *(3)*. In spite of these toxic reactions, however, viomycin is still used clinically in cases in which the tuberculosis microorganism has become resistant to streptomycin.

Analysis of the crystalline sulfate, hydrochloride, picrate, and reinechate salts of viomycin indicated an empirical formula of $C_{18}H_{31-33}N_9O_8$ for the free base *(3,4)*. The sulfate salt melts at 252° with decomposition and has a specific rotation of -39.8° in water *(2)*. Later, using a diaphragm-cell diffusion technique on the sulfate salt, a molecular weight of 772 was indicated for the sulfate salt. This result, together with analytical data and chemical evidence, suggested a molecular formula of $C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$ (Mol. wt. 836) *(5)*.

Viomycin is a strong base, and has pKa* values of 8.3, 10.3, and 12.2 in water. The pKa values of 8.3 and 10.3 are due to amino groups and that of 12.2 is due to a guanidine function. The absence

of a pKa value lower than 8.3 indicates that viomycin does not have a free carboxyl group *(5)*. Van Slyke primary amino nitrogen determinations indicated that 1.22 primary amino groups in viomycin react in 2.5 minutes, 1.98 groups react in 15 minutes, and 2.16 groups react in one hour (assumed molecular weight, 836) *(6)*.

Viomycin exhibits one strong absorption in the ultraviolet region, the position and intensity of which depend on the pH of the solution. The peak occurs at 268 mμ (ε, 23,300) in 0.1 N hydrochloric acid, at 268.5 mμ (ε, 22,900) in neutral solution, and at 282.5 mμ (ε, 14,600) in 0.1 N sodium hydroxide. Determination of the ultraviolet spectra in solutions of pH 10, 11, 12, 13 and 14 indicated two isobestic points at 235 and 281 mμ, thus indicating that one dissociating group was involved with the chromophore *(5,7)*. It was shown that the group involved has a pKa value of 12.4, which indicated that the guanidine group present in viomycin is involved in the ultraviolet chromophore *(5)*. Catalytic hydrogenation of viomycin did not change the ultraviolet spectrum *(5)*. When viomycin is heated in 0.1 N hydrochloric acid at 100°, the ultraviolet absorbance decreases gradually and after six hours disappears. A lyopholized sample of this solution shows ultraviolet absorption, however *(8)*.

Viomycin shows positive Sakaguchi and ninhydrin tests, but negative Benedict's, Fehling's, and maltol tests *(1,2,6)*. These results

---

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

indicated the presence of guanidine and amino groups. In addition viomycin gives a positive biuret test *(4)*. This fact together with resistance to mild acid hydrolysis indicated that viomycin has a peptide function. The presence of a carbohydrate moiety appeared unlikely on the basis of color tests and failure of viomycin to react with periodic acid *(1,2)*.

Hydrolysis of viomycin sulfate with 1 N hydrochloric acid for eleven days at 37° reduced the microbiological activity to 25 per cent of the original value, but did not release appreciable amounts of amino acids *(4)*. However, upon vigorous acid hydrolysis in 6 N hydrochloric acid or 6 N sulfuric acid at 100°, the ultraviolet chromophore of viomycin was destroyed and amino acids were released *(4)*. Carbon dioxide, ammonia, and urea were identified in the hydrolysis mixture as well as four ninhydrin positive components which could be isolated from the hydrolysis mixture by using cation exchange chromatography with Zeo Rex (H^+), a phenolic methylenesulfonic acid type ion-exchange resin *(4)*. Urea was isolated as the dixanthydryl derivative. The four ninhydin positive components that were isolated were shown to be L-serine, L-α,β-diaminopropionic acid, L-β-lysine (L-3,6-diaminohexanoic acid), and a mixture of guanidino compounds. L-serine was identified by optical rotation, analysis, preparation of the N-2,4-dinitrophenyl derivative, and by the identity of the infrared absorption spectrum with that of authentic L-serine *(4)*. L-α,β-diaminopropionic acid was identified as the monohydrochloride salt by analysis, van Slyke amino nitrogen determination, and ninhydrin carbon dioxide determination. The L-configuration was indicated for α,β-diaminopropionic acid on the

basis of its optical rotation and the optical rotation of the N,N'-dibenzoyl derivative *(5)*. Analyses of the crystalline hydrochloride, sulfate, picrate, and p-hydroxyazobenzene p-sulfonate salts of L-β-lysine indicated that the compound was identical with the isomer of lysine isolated from streptothricin and streptolin hydrolysates and shown to be identical with synthetic L-3-6-diaminohexanoic acid *(4,9)*. The mixture of guanidino compounds was found to contain a major component which was isolated in crystalline form and named viomycidine, $C_6H_{10}N_4O_2 \cdot HCl$ *(10,15)*.

Hydrolysis of viomycin with 0.43 N barium hydroxide or 0.5 N lithium hydroxide at 95° for three days yielded ammonia, carbon dioxide, and a number of amino acids as identified by two-dimensional paper chromatography: β-lysine, serine, α,β-diaminopropionic acid, and alanine *(5)*. Since alanine does not occur in the acid hydrolysate of viomycin, the basic hydrolysate was subjected to preparative paper chromatography. The section corresponding to alanine was eluted with water and a compound was obtained which had a nuclear magnetic resonance spectrum in deuterium oxide that was identical to a spectrum of authentic alanine determined in a similar solution. Furthermore, the infrared spectrum of the 2,4-dinitrophenyl derivative of the compound was identical with a spectrum of the 2,4-dinitrophenyl derivative of an authentic sample of DL-alanine *(11)*. The presence of alanine in the basic hydrolysate of viomycin and its absence from the acid hydrolysate was attributed to the attack of base on serine to give alanine, since it could be shown that treatment of serine under the same conditions used for the basic hydrolysis of viomycim yielded a mixture of

serine and alanine *(11)*.

Hydrolysis of viomycin with 6 N hydrochloric acid at room temperature released urea, serine, and β-lysine during the first day. The release of β-lysine continued up to the fourth day, and the release of serine continued up to ten days. After eleven days a complex pattern of ninhydrin spots was observed, but no discrete spot corresponding to α,β-diaminopropionic acid or viomycidine could be observed *(6)*.

Oxidation of viomycin with potassium permanganate solution resulted in the destruction of the ultraviolet chromophore and the consumption of four moles of permanganate per three moles of viomycin (assumed molecular weight of viomycin sulfate, 774). No apparent fragmentation of viomycin took place and no carboxylic acid groups were produced in the oxidation. Furthermore, paper chromatography indicated that the 6 N hydrochloric acid hydrolysate of the oxidation product of viomycin contained the same compounds as the 6 N acid hydrolysate of viomycin itself *(5)*.

Treatment of a solution of viomycin in cold aqueous acetic acid with ozone followed by treatment with hydrogen peroxide resulted in the destruction of the ultraviolet chromophore. Analysis of the product of ozonolysis by paper chromatography showed only one ninhydrin-positive spot, thus indicating that the site of oxidation is in a cyclic residue in the viomycin molecule. Hydrolysis of the ozonolysis product with 6 N hydrochloric acid at 95° for six hours released serine, β-lysine, α,β-diaminopropionic acid, viomycidine, guanidine, and an unknown guanidino compound *(6,11)*. The release of guanidine suggested that the guanidine unit in viomycidine might be present as an ethylenic

group *(5,6)*.

Early analysis of the salts of viomycin suggested an empirical formula $C_{18}H_{33}N_9O_8 \cdot 3/2\ H_2SO_4$ (molecular weight, 650.65) for viomycin sulfate *(4)*. Later the molecular weight of viomycin sulfate was determined to be 772 using a diaphragm-cell diffusion technique and viomycin sulfate was assigned the molecular formula $C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$ (Formula I, molecular weight, 836) *(5)*. It was noted, however, that the addition of the formulas of the known hydrolysis fragments gave only 20 carbon atoms *(5,6)*:

Serine	$C_3H_7NO_3$
α,β-Diaminopropionic Acid	$C_3H_8N_2O_2$
β-Lysine	$C_6H_{14}N_2O_2$
Viomycidine	$C_6H_{10}N_4O_2$
Urea	CH_4N_2O
Carbon Dioxide	CO_2
Ammonia	H_3N
	$C_{20}H_{46}N_{12}O_{12}$

Subtraction of five moles of water, one for each carboxyl group and one for carbon dioxide gives Formula II, $C_{20}H_{36}N_{12}O_7 \cdot 3/2\ H_2SO_4$ (molecular weight 707.73). More than one mole of serine could be isolated from the hydrolysate of viomycin per mole of viomycin hydrolyzed, thus indicating that viomycin contains two serine residues *(5)*. The ratio of amino acids resulting from acid hydrolysis was determined to be L-serine:L-α,β-diaminopropionic acid:L-β-lysine:viomycidine = 2:1:1:1 *(8,12)*. Addition of a second serine fragment, less a molecule

of water gives Formula III, $C_{23}H_{41}N_{13}O_9 \cdot 3/2\ H_2SO_4$ (molecular weight, 790.81). Due to lack of agreement between analytical data for nitrogen and molecular formulas obtained by adding in one mole of ammonia, doubt has existed as to the origin of the ammonia in the hydrolysate of viomycin. When viomycin was hydrolyzed in 6 N hydrochloric acid at 95° for six hours and the total hydrolysate analyzed by an amino acid analyzer, the results revealed an actual ratio of 1.00 mole of urea, 0.110 mole of ammonia, and 0.145 mole of viomycidine *(11)*. When urea was hydrolyzed under the same conditions, 0.073 mole of ammonia was released per mole of urea *(11)*. Since the release of a small amount of ammonia from each amino acid is also possible, it was concluded that ammonia is an artifact in the hydrolysate. When a mole of ammonia is subtracted from Formula III, Formula IV, $C_{23}H_{38}N_{12}O_9 \cdot 3/2\ H_2SO_4$ (molecular weight, 773.77), results for viomycin sulfate *(5,6)*.

Table 1 compares the calculated values for the four suggested molecular formulas with reported analytical results for carbon, hydrogen, nitrogen and sulfur for viomycin sulfate. The ratio of carbon to nitrogen is also shown in Table 1 since this value is independent of the dryness of the sample or the stoichiometry of the sulfate salt.

Johnson and co-workers *(12,13)* reported the isolation of four peptides from partial hydrolysis of viomycin. Peptide A could be isolated as the crystalline tripicrate. This peptide showed positive ninhydrin and Sakaguchi reactions, pKa's of 9.9 and 11.2, and gave ultraviolet absorptions similar to viomycin, λ_{max} 275 mμ (ε, 5100) at pH of 1 and λ_{max} 295 mμ (ε, 3600) at a pH of 10. Hydrolysis of Peptide A with boiling 12 N hydrochloric acid gave α,β-diaminopropionic acid

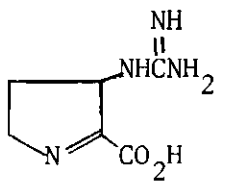
Table 1. Per Cent Composition of Viomycin Sulfate

	C	H	N	S	C/N
Formula I					
($C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$)	35.92	5.67	20.22	5.74	1.80
Formula II					
($C_{20}H_{36}N_{12}O_7 \cdot 3/2\ H_2SO_4$)	34.11	5.58	23.89	6.83	1.44
Formula III					
($C_{23}H_{41}N_{13}O_9 \cdot 3/2\ H_2SO_4$	34.92	5.61	23.03	6.08	1.52
Formula IV					
($C_{23}H_{38}N_{12}O_9 \cdot 3/2\ H_2SO_4$)	35.70	5.34	21.72	6.22	1.70
Reported (1)	37.19	5.86	20.61	5.88[a]	1.80
Reported (2)	35.83	5.77	21.08	5.34	1.70
Reported (4)	35.89	5.52	21.12	5.79	1.70
Reported (5)	34.22[b]	5.73[b]	21.85[b]	4.73[b]	1.56[b]
Reported (6)	35.01	6.19	21.01	5.73	1.67
Average of Reported	35.71	5.83	21.19	5.51	1.69

[a]Determined as sulfate

[b]Not corrected for residue after ignition (1.25 per cent).

and viomycidine in a 1:1 molar ratio. Hydrolysis of the 2,4-dinitrophenyl derivative of Peptide A gave bis-2,4-dinitrophenyl-α,β-diaminopropionic acid *(13)*. Peptide B, which was obtained from both acid and base hydrolysates of viomycin, gave equimolar amounts of β-lysine and serine on further hydrolysis; and the bis-2,4-nitrophenyl derivative of Peptide B was reported to give mono-ε-2,4-dinitrophenyl-β-lysine and N-2,4-dinitrophenylserine on hydrolysis *(12)*. Peptide C was isolated in very small amounts after hydrolysis of viomycin with 0.1 N lithium hydroxide and it was reported to give equimolar amounts of viomycidine and β-lysine on further hydrolysis *(12)*. Peptide D, which was also isolated from the 0.1 N lithium hydroxide hydrolysate, although in better yield than Peptide C, formed a crystalline tripicrate and gave approximately equimolar amounts of β-lysine, viomycidine, and serine on further hydrolysis. The 2,4-dinitrophenyl derivative of Peptide D was also reported to give mono-ε-2,4-dinitrophenyl-β-lysine and N-2,4-dinitrophenylserine upon hydrolysis *(12)*. Based on structure I for viomycidine *(13)*, Peptide A was proposed to have structure II *(14)*. Peptides B and D were proposed to have structures III and IV, respectively *(12)*. Based partially on the fact that a



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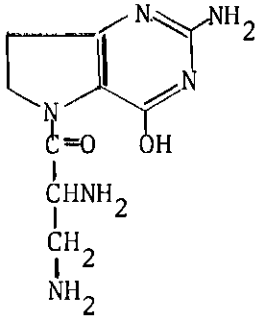

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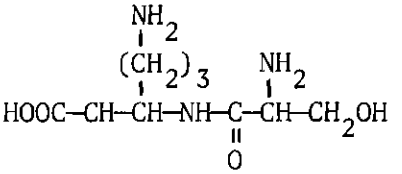
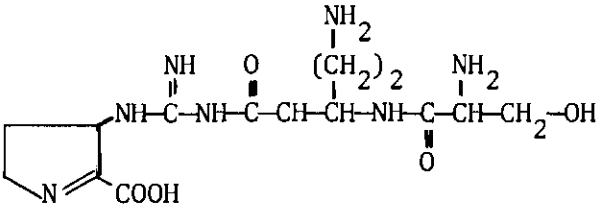


III

compound believed to be L-serine allophanate(V) could be isolated after the potassium permanganate oxidation of viomycin, a partial structure for viomycin(VI) was proposed *(12)*.



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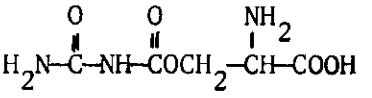

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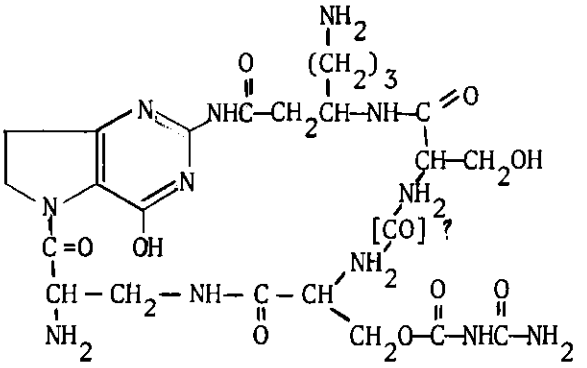


VI

Shortly thereafter, Dyer and co-workers *(15)* reported findings which were not in complete agreement with those of Johnson and co-workers and which led to the proposal of a new structure for viomycin. It was first pointed out that since viomycidine, as obtained from viomycin hydrolysate, was optically active then structure II must be rejected as an integral structural unit of the viomycin molecule *(15)*.

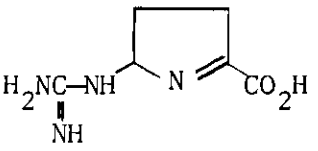
Hydrazinolysis of viomycin did not give a free amino acid, thus confirming the absence of a free carboxyl group in viomycin. Hydrolysis of bis-2,4-dinitrophenylviomycin gave only one 2,4-dinitrophenyl derivative, bis-2,4-dinitrophenyl-β-lysine, thus indicating that the two free amino groups in viomycin are those of the β-lysyl residue *(6)*.

Hydrolysis of viomycin with 0.1 N hydrochloric acid at 95° for six hours released one equivalent of urea. No other amino acid was released and the resulting product, desureaviomycin, was isolated and characterized *(5,6)*. Complete acid hydrolysis of desureaviomycin gave all of products of hydrolysis of viomycin except urea. Hydrazinolysis of desureaviomycin indicated that it has a free seryl carboxyl group *(6)*. Hydrolysis of bis-2,4-dinitrophenyldesureaviomycin gave bis-2,4-dinitrophenyl-β-lysine as the only 2,4-dinitrophenyl derivative. This showed that no primary amine groups are released in the transformation of viomycin to desureaviomycin *(6)*.

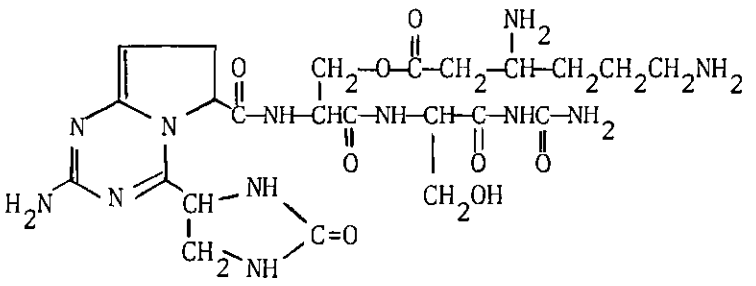
Upon treatment of desureaviomycin with the enzyme carboxypeptidase at pH 7 for two days, serine was released. The other product was isolated, characterized, and named viomycinic acid *(6)*. Hydrazinolysis of viomycinic acid indicated that the carboxyl group of a seryl residue was free in viomycinic acid. Complete hydrolysis of viomycinic acid released all of the components of the viomycin hydrolysate except urea. Hydrolysis of bis-2,4-dinitrophenylviomycinic acid gave only bis-2,4-dinitrophenyl-β-lysine *(6)*.

Hydrolysis of viomycin sulfate in boiling water for three weeks gave a dipeptide that gave on complete hydrolysis approximately

equimolar amounts of serine and β-lysine *(15)*. Although this dipeptide was considered to be identical to Peptide B obtained by Johnson and co-workers, it was formulated as arising from an O-β-lysylseryl unit rather than an N-β-lysylseryl unit. Based on the above evidence and structure VII for viomycidine *(17)*, structure VIII was proposed for viomycin *(15)*. The inconsistencies between the structure VIII and some of the results of Johnson and co-workers were attributed to the fact that some of the peptides isolated by Johnson and co-workers may have been mixtures. It



VII



VIII

was also pointed out that the fact that Johnson and co-workers reported mono-ε- rather than bis-2,4-dinitrophenyl-β-lysine could possibly be

explained by noting that under the usual conditions of hydrolysis, boiling 6 N hydrochloric acid, bis-2,4-dinitrophenyl-β-lysine decomposes to some extent to a mono-2,4-dinitrophenyl-β-lysine and 2,4-dinitrophenol. On the other hand very little decomposition occurs if the hydrolysis is carried out in 12 N hydrochloric acid at 95° in a pressure bottle *(15)*.

More recently, Kitagawo et al. *(8)* reported that partial hydrolysis of viomycin in 1 N hydrochloric acid at 100° for six hours resulted in a hydrolysate from which a number of peptides could be isolated by column chromatography. Peptide III $[\alpha]_D^{27°}$ = -2° (0.1% in H_2O), gave no ultraviolet absorption but gave a positive Sakaguchi reaction. Complete hydrolysis of peptide III gave serine, diaminopropionic acid and viomycidine. Hydrazinolysis of peptide III gave viomycidine as a free amino acid. Hydrolysis of the bis-2,4-dinitrophenylpeptide III yielded viomycidine, N-2,4-dinitrophenylserine, and a mono-2,4-dinitrophenyldiaminopropionic acid that was assigned the α-amino-β-2,4-dinitrophenylaminopropionic acid structure by comparison with the synthetic compound. The amino acid sequence of peptide III can thus be inferred to be seryl-diaminopropionyl-viomycidine, where the β-amino group of diaminopropionic acid is free *(8)* .

Peptide II, $[\alpha]_D^{17}$ = -38° (1% in H_2O), gave λ_{max} 268 mμ(log ε, 4.12) and a positive Sakaguchi reaction. Complete hydrolysis of peptide II gave serine, diaminopropionic acid, β-lysine, and viomycidine in the ratio 2:1:1:1, respectively. Hydrazinolysis of peptide II gave serine as a free amino acid. Acid hydrolysis of the tris-2,4-dinitrophenylpeptide II gave free serine, free viomycidine, bis-2,4-dinitro-

phenyl-β-lysine and α-amino-β-2,4-dinitrophenylaminopropionic acid. The amino acid sequence of peptide II was thus inferred to be: β-lysyl-seryl-diaminopropionyl-viomycidyl-serine where the β-amino group of diaminopropionic acid is free *(8)* .

Three peptides, Peptides Ia, b, and c, could be isolated which had identical amino acid sequences but which showed different ultraviolet absorption, R_F values on thin-layer chromatography and R values on electrophoresis. Peptide Ic could also be obtained along with urea under the same conditions used by Dyer and co-workers *(6,15)* to prepare desureaviomycin. The amino acid components, N-terminal amino acid, and C-terminal amino acid of peptide Ic are identical to those of desureaviomycin. However, desureaviomycin was reported to have no ultraviolet absorption while peptide Ic does have an ultraviolet absorption. Complete hydrolysis of each member of the peptide I group gave serine, diaminopropionic acid, β-lysine, and viomycidine in ratios 2:1:1:1, respectively. Acid hydrolysis of the bis-2,4-dinitrophenyl-peptide I group gave bis-2,4-dinitrophenyl-β-lysine plus all of the above mentioned amino acids except β-lysine. Hydrazinolysis of all of the peptide I group gave serine as a free amino acid. The amino acid sequence of the peptide I group was thus inferred to be: β-lysyl-seryl-diaminopropionyl-viomycidyl-serine where the β-amino group of diaminopropionic acid is tied up some way *(8)*.

Hydrazinolysis of viomycin did not give a free amino acid, and hydrolysis of bis-2,4-dinitrophenylviomycin gave bis-2,4-dinitrophenyl-β-lysine as the only 2,4-dinitrophenyl derivative *(8)*, both results being the same as those reported by Dyer and co-workers *(15)*. The amino

acid sequence of viomycin was concluded to be: β-lysyl-seryl-diaminopropionyl-viomycidyl-seryl-urea (IX) where the β-amino group of diaminopropionic acid is not free *(8)*. This amino acid sequence is not consistent with either structure VI or structure VIII.

Neither serine nor viomycinic acid could be obtained by the action of carboxypeptidase A on peptide Ic *(8)*. It should be noted that all of the results reported by Dyer and co-workers *(15)* are consistent with the proposed amino acid sequence for viomycin, structure IX, with the exception of the preparation and reactions of viomycinic acid.

It was discovered early that the acid hydrolysate of viomycin contained a number of strongly basic compounds that gave positive Sakaguchi tests. These compounds could be separated as a mixture from the other hydrolysis products by ion-exchange chromatography *(4)*. Carbon chromatography of this basic mixture yielded a crystalline compound which was named viomycidine *(16, 19)*. Analysis of the monohydrochloride salt of viomycidine indicated the formula $C_6H_{10}N_4O_2$ for the free base of viomycidine. The specific rotation of viomycidine varied with pH, being -21.2° in 2 N hydrochloric acid, -83.2° in water, and -155.8° in 1 N sodium hydroxide solution *(10)*. Van Slyke amino nitrogen determination showed no primary amino groups. Further analysis also indicated no N-methyl, O-methyl, or C-methyl groups *(10)*.

Viomycidine showed pKa values of 2.8, 5.87, and 13.4 in 66 per cent N,N-dimethylformamide and 5.50 and 12.6 in water *(10)*. The function corresponding to pKa of 2.8 in 66 per cent DMF was too acidic to be accurately determined in water by the method used but its pKa was

estimated to be 1.3 in water *(16)*. This group was judged to be a carboxylic acid group near two basic centers as it was considered unlikely that there could be any other group in viomycidine with the observed pKa values *(16)*.

Viomycidine gave a pink color with the Sakaguchi reagent, thus indicating the presence of a monosubstituted guanidine group. The presence of a guanidine group is also consistent with the pKa values of 12.6 in water and 13.4 in 66 per cent DMF. Viomycidine gave a positive Weber test, a purple-pink color with ninhydrin reagent, a negative result with Tollen's reagent, and no color change with Benedict-Behre reagent. When heated under the conditions for the transformation of creatine into creatinine, it still did not give a positive test with Benedict-Behre reagent but did still give a positive Sakaguchi test. These results indicated that no cyclization to the creatinine-type linkage had occurred. Viomycidine gave a negative ferric chloride test, which indicated the absence of an enolic group *(10)*.

The infrared spectrum of viomycidine hydrochloride showed a large number of absorptions among which could be assigned those for N-H stretching (2.98μ and 3.18μ), C-H stretching (3.50), $-CH_2-$ deformation (6.87μ), and carboxylate anion vibrations (6.20μ or 6.33μ). In addition two bands characteristic of monosubstituted or N,N-disubstituted guanidines occur at 5.91 and 6.06 *(10)*.

The nuclear magnetic resonance spectrum of viomycidine hydrochloride in deuterium oxide solution revealed absorptions at τ7.43 (two protons, triplet, J = 2.1 cps), 5.38 (two protons, closely spaced

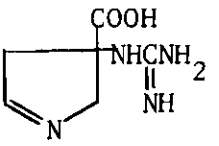
multiplet), and 4.37 (one proton, triplet, J = 2.2 cps) *(11, 15)*. The spectrum in trifluoroacetic acid solution revealed the same proton ratio (2:2:1) for the hydrogens bonded to carbon with each of the peaks at a slightly lower τ value than the corresponding peak in deuterium oxide solution. In addition, three more absorption peaks were observed: at τ3.00 (two protons), 1.92 (one proton), and 1.48 (one proton) with none of these peaks split *(16)*.

When viomycidine was heated with concentrated hydrochloride acid for six days, it was partially converted into at least five other ninhydrine positive compounds, some of which also gave positive Weber reactions. An appreciable amount of viomycidine still remained after this length of time *(10)*.

The formula for viomycidine indicated four rings and/or double bonds. The carboxylic acid group and the guanidine group account for two double bonds. Viomycidine absorbed one mole of hydrogen in the presence of platinum and acetic acid, thus indicating that viomycidine has a cyclic structure with one double bond in addition to the carboxyl and guanidine groups *(10, 16)*. Since viomycidine was reasonably stable to acid, a five-membered ring was indicated as opposed to a three- or four-membered ring. When the ultraviolet spectrum was determined with a pH of 9.80 in one cell and a pH of 3.82 in the other, both containing the same concentration of viomycidine hydrochloride, an absorption at 212 mμ (ϵ, 2,530) was observed *(10)*. The presence of such a differential ultraviolet spectrum indicated that the group in viomycidine with pKa of 5.50 in water was a tertiary amine *(10)*.

It was noted that the presence of a Δ^1-pyrroline ring would account for the tertiary amine as well as the five-membered ring and double bond indicated for viomycidine. Viomycidine gave a positive test with the o-aminobenzaldehyde reagent, a test typical for imines and Δ^1-pyrrolines.

On the basis of the above physical and chemical data, structure X, 4-guanidino-Δ^1-pyrroline-4-carboxylic acid, was considered for viomycidine *(16)*. Structure X was eliminated from consideration when it



X

was observed that in the barium hydroxide hydrolysis of viomycidine at 95° for 77 hours pyrrole-2-carboxylic acid was formed in 22 per cent yield along with 2.7 equivalents of ammonia *(16)*.

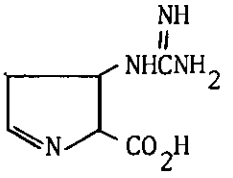
When viomycidine was hydrolyzed with hot concentrated sodium hydroxide at 100°, 2-aminopyrimidine, glycine, and pyrrole-2-carboxylic acid could be obtained and identified *(16)*.

Preparation of the 2,4-dinitrophenyl derivative of viomycidine gave a yellow crystalline compound that melted at 171.5-172.5° with decomposition. Elemental analysis indicated a formula of $C_{12}H_{12}N_6O_6 \cdot 2H_2O$ for the derivative. 2,4-Dinitrophenylviomycidine gave a pink

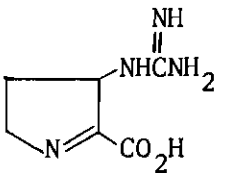
color with Weber reagent but a negative test with ninhydrin reagent *(16)*.

The acetyl derivative of viomycidine was prepared using aqueous ethanolic acetic anhydride. The acetyl derivative was a white crystalline solid that melted at 256-257° with decomposition and had an optical rotation of +41° in water. Elemental analysis indicated an empirical formula of $C_8H_{12}N_4O_3$. Acetylviomycidine gave an orange color with Weber reagent but it gave negative tests with the ninhydrin, o-aminobenzaldehyde, Sakaguchi, and Benedict-Behre reagents *(11)*. When acetylviomycidine was treated with ozone followed by oxidation with hydrogen peroxide and acid hydrolysis, the resulting mixture contained four ninhydrin and Weber positive substances, but showed no viomycidine by paper chromatography *(16)*.

After rejection of structure X for viomycidine, and based on the fact that pyrrole-2-carboxylic acid was obtained from basic hydrolysis of viomycidine, two possible structures for viomycidine were suggested, 4-guanidino-Δ^1-pyrroline-5-carboxylic acid (XI) and 3-guanidino-Δ^1-pyrroline-2-carboxylic acid (I) *(16)*. Due to the absence of a



XI



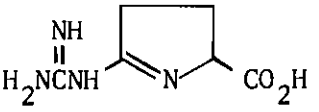
I

low-field proton in the nuclear magnetic resonance spectrum characteristic of a proton bonded to the carbon of a carbon-nitrogen double bond, structure XI was rejected *(16)*. The nuclear magnetic resonance spectra of various model compounds were used to estimate the absorption positions that might be expected for the protons in structures XI and I. The estimates for structure I were in much better agreement with the observed absorption positions than were the estimates for structure XI. On this basis structure I was proposed as the structure of viomydicine *(16)*.

Johnson and co-workers *(12,14)* accepted structure I for viomycidine and on the basis of this structure proposed a structure for their peptide A (II), a dipeptide they isolated from hydrolysate of viomycin.

Viomycidine was acetylated with hot acetic anhydride in pyridine. Ozonlysis of this product followed by oxidation with hydrogen peroxide and hydrolysis in hydrochloric acid yielded crystalline DL-aspartic acid and guanidine as the only isolable products. The DL-aspartic acid was isolated in 53 per cent yield and was purified by carbon chromatography. It was identified on the basis of the fact that its infrared spectrum was superimposable on that of an authentic sample of DL-aspartic acid and its nuclear magnetic resonance spectrum in 0.5 N hydrochloric acid was identical to that of authentic DL-aspartic acid in the same solvent. The fact that racemic aspartic acid rather than the expected optically active aspartic acid was obtained was attributed to the use of hot pyridine at one state of the reaction sequence. If structure I had been correct, the reaction sequence carried out on

acetylviomycidine could not have yielded aspartic acid. The structure I was excluded and structure VII, 2-guanidino-Δ^1-pyrroline-5-carboxylic acid, was proposed as the correct structure for viomycidine *(11,17)*.



VII

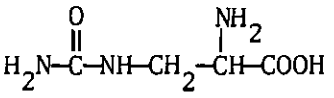
As viomycidine is more dextrorotatory in acid solution than in water, application of the Clough-Lutz-Jurgenson rule suggested the L (or R) absolute configuration for the asymmetric center present in viomycidine *(16)*.

No direct information is available concerning the biosynthesis of viomycin, although some information is available on the hydrolysis products of viomycin.

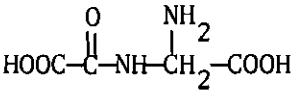
Since serine is one of the common amino acids, it should be available to the organism producing viomycin either from the growth medium or by well-known biosynthetic pathways *(18)* and could be incorporated intact into the viomycin molecule.

α,β-Diaminopionic acid and several of its derivatives have been isolated from a variety of natural sources. Albizzine (α-amino-β-ureidopropionic acid (XII) has been isolated from several species of Mimosaceae *(19)* and β-N-oxalyl-α,β-diaminopropionic acid (XIII) from

Lathyrus sativus *(20)*. L-α,β-Diaminopropionic acid has been isolated in high concentration from seeds of Vicia baicalensis *(21)*, and has also been isolated from the hydrolysate of the antibiotic edeine *(22)*. D-α,β-Diaminopropionic acid has been found in the digestive fluid of the fifth instar of the larvae of Bombyx mori *(23)*.

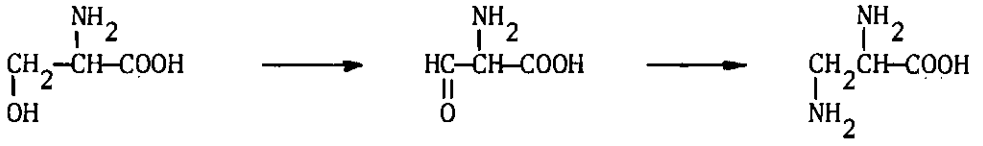


XII



XIII

The biosynthesis of albizzine was studied in Albizzia loparetha *(24)*. Glycine-1-^{14}C, glycine-2-^{14}C, serine-1-^{14}C, and serine-3-^{14}C were incorporated into albizzine to a significant extent. Glyoxylate-U-^{14}C and formaldehyde-^{14}C were incorporated to some extent, probably as was pointed out, via serine. Uric acid-2-^{14}C was incorporated slightly, but carbon dioxide-^{14}C, formic acid-^{14}C, urea-^{14}C, uricil-2-^{14}C, and arginine-amidine-^{14}C gave only negligible incorporation. The following pathway for the biosynthesis of α,β-diaminopropionic acid was suggested *(24)*.



L-β-Lysine (3,6-diaminocaproic acid) has been derived by acid hydrolysis from a number of antibiotics other than viomycin including streptolin AB *(25)*, steptothricin *(26)*, roseothricin *(27)*, and geomycin *(28)*. Studies of L-lysine fermentation by an unidentified species of Clostridium revealed that L-β-lysine could be made to accumulate under certain conditions *(29)*. It was determined that β-lysine accumulates on deletion of cofactors (nicotinamide-adenine dinuclotide and adenosine-5'-diphosphate) required for lysine fermentation; that is formed from lysine by a readily reversible reaction; that it is fermented more rapidly than lysine when suitable cofactors are added; and that the addition of lysine does not markedly inhibit β-lysine fermentation. It was concluded that β-lysine lies on or very close to the path of lysine fermentation to ammonia and acetic and butyric acids. α-Ketoglutarate and coenzyme A were found to be required as cofactors in the conversion of lysine to β-lysine *(29)*. It was later shown that the fermentation of lysine to ammonia, acetic acid and butyric acid by Clostridium sticklandii involves initially two successive amino group migrations, forming first β-lysine, followed by conversion of β-lysine to 3,5-diaminohexanoic acid *(30)*. It was found that a cobamide coenzyme was required for the second conversion, but not the first, which appears instead to require the participation of pyridoxal phosphate *(31)*. Furthermore, it was shown that both the migration of the α-amino group to the β-position and the migration of the ε-amino group to the γ-position does not involve exchange with free ammonia nitrogen. This result excludes an α,β-unsaturated intermediate formed by ammonia elimination followed by readdition of

ammonia in the β-position. Also excluded would be any intermediate transfer of the amino group to a keto group (transamination) which would lead to exchange of the nitrogen with free ammonia. It was suggested that the reaction mechanism might involve the intermediate formation of an aziridine ring, but it was pointed out that there was no evidence eitherto support or eliminate this possibility *(31)*.

Viomycin is a peptide that yields upon complete acid hydrolysis theamino acids L-serine, L-α,β-diaminopropionic acid, L-β-lysine, and viomycidine as well as urea, carbon dioxide, and ammonia. The purposes of this research were initially twofold: First, to synthesize viomycidine by a rational synthetic approach, both to provide conclusive proof of its structure and to provide a source of cold carrier for any degradation reactions to be carried out on radioactively labelled viomycidine. Second, to determine the biological precursors of the unusual amino acid fragments of viomycin: L-α,β-diaminopropionic acid, L-β-lysine, and viomycidine.

CHAPTER II

EXPERIMENTAL

Apparatus and Techniques

Unless otherwise stated, all concentrations and evaporations were performed using a Rinco (Model VE-1000A) rotary evaporator modified to an all glass system and used at water aspirator vacuum and steam bath temperature. Drying of solutions and extracts in organic solvents was accomplished, unless otherwise stated, but the addition of anhydrous magnesium sulfate. The drying agent was removed by gravity filtration and washed thoroughly with several fresh portions of solvent.

All melting points were performed using a Köfler hot stage and are corrected. Microanalyses were performed by Bernhardt Laboratories (Mülheim, West Germany).

Qualitative color test used included: ninhydrin *(32)*, Sakaguchi *(32)*, Nessler *(10)*, and p-dimethylaminobenzaldehyde *(32)*. Paper chromatography and thin layer chromatography (TLC) were performed as described elsewhere *(10,11)*. Unless otherwise noted, the solvent system used in TLC was t-butyl alcohol:acetic acid:water, 2:1:1 (v/v)(BAW). Spray reagents most frequently used in paper chromatography and TLC were ninhydrin and Weber. Their preparation and the interpretation of results are as given previously *(32)*.

Ion-exchange resins used in this work were regenerated and used as described previously *(10,33)*. The following abbreviations are used:

IR-45(OH^-), IR-45(Cl^-), and IR-45(Br^-) for Amberlite anion exchange resin 45 in the hydroxyl, chloride, and bromide phase, respectively; IRC-50(H^+) for amberlite cation exchange resin 50 in the hydrogen phase; and Dowex 50(H^+) for Dowex 50W X-8 (100-200 mesh, Baker reagent 1930) in the hydrogen phase.

Carbon chromatography was performed using pretreated (3% oleic acid) Darco G-60 (Atlas Powder Co.) and acid-washed Celite (Johns-Manville Corporation) prepared and used as described previously *(10)*.

Optical rotations were determined using a Bellingham and Stanley Model No. 397619 polarimeter with the sodium D line as a light source. A Perkin Elmer Model 137 recording spectrophotometer was used to obtain infrared spectra. Potassium bromide pellets were prepared by grinding about 1.0 mg of the sample in about 200 mg of potassium bromide. The pellets were pressed at 2500 psig. Nuclear magnetic resonance (n.m.r.) spectra were determined using a Varian Model A-60 spectrometer. Chemical shift values are reported in τ units ($\tau = 10-\delta$). Tetramethylsilane (TMS) or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard. Concentrations are given as per cent by weight. All mass spectral data were determined using a Varian Model M-66 mass spectrometer.

A Wilsback Model T-408 ozone generator was used in all ozonolyses. Ozone was generated at 75 volts with a flow rate of 0.75 standard liters per minute and a pressure of 8 psig.

Techniques for Liquid Scintillation Counting

All counting of radioactive samples was done with a Packard Tri-Carb Model 3375 Liquid Scintillation Spectrometer. Unless otherwise indicated, all samples were counted using the green channel with the ^{14}C

channel selector in. This setting automatically sets the amplification and discriminator controls to give a maximum count when counting ^{14}C-labelled samples as determined by the factory. It was not possible to significantly improve the counting by manually adjusting the amplification and window openings. The print-out was normally set to print AES ratio, sample number, time, gross counts for the green channel, and net counts per minute (cpm) for the green channel on the data sheets. The following dial settings were normally made:

Preset count	900,000
Low-level reject	off
Number of counts	1
Number of cycles	∞

Background was determined using samples made up as close as possible to the samples to be counted but without any radioactive material. These background samples were counted for relatively long counts (up to 500 minutes) using the same dial settings that would be used for the radioactive samples except that the background subtraction dial was set to zero. After the background count was determined, it was dialed onto the background subtraction dial, and the radioactive samples were then counted. Since radioactive decay events occur randomly, the degree of error in a count may be computed by the laws of statistics. Statistically it may be shown that the amount of error is determined entirely by the total number of counts recorded. If the total number of counts is 10,000, then it may be computed that the observed count rate will vary from the true average count rate by more than one per cent at most in only three out of ten count rate measurements *(34)*. For this reason, samples were normally made up to give a

count rate of 1,000 cpm or more so that a ten minute count period would suffice to give the desired 10,000 total counts.

Two different scintillation solutions were used routinely depending on the solubility characteristics of the sample to be counted. For toluene-soluble samples, a toluene scintillation solution was used. The toluene scintillator solution was made up by dissolving five grams of 2,5-diphenyloxazole (PPO) as the primary scintillator and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) as the secondary scintillator per liter of redistilled toluene. For water-soluble samples a toluene-Triton X-100 scintillation solution (tT21) was used *(35)*. The tT21 scintillation solution was prepared by mixing together four liters of redistilled toluene, two kilograms of Triton X-100 (Reg. Trademark of Rohme & Haas, Inc.) purified for liquid scintillation counting (Packard Instrument Company, Inc., Cat. No. 6008083), 13.872 g. of PPO, and 0.347 g. of POPOP. Normal background counts for the toluene scintillation solution samples ranged from 28 to 31 cpm and for the tT21 scintillation solution samples the range was 36 to 39 cpm.

Counting efficiencies of samples were determined by two different methods. When a large series of samples of similar chemistry were to be counted, a quenching curve was determined relating the automatic external standardization (AES) ratio to the counting efficiency. To relate the AES ratio to the counting efficiency, two series of control samples were made up. The first series of control samples (the background control samples) contained the same type of specimen-scintillation solution that was typical of the experimental samples to be counted, but they contained no radioactivity. The first control sample of this series was made up

with none of the expected quenching substance added. The two or three additional control samples in this series contained increasing concentrations of the expected quenching substance up to and beyond the maximum concentration of the quenching substance expected to be found in the experimental samples. This series of control samples was used to determine typical background counts for both quenched and unquenched samples. The second series contained six or seven control samples, each made up with the specimen-scintillation solution typical of the experimental samples to be counted. Each sample contained a different amount of the expected quenching substance so that the series covered the entire range from unquenched to nearly completely quenched. Each sample also contained a known amount of benzoic-^{14}C acid (the same activity was added to each sample to within $\pm$1%). After counting both series of control samples, the activities obtained for the background samples were correlated with the amount of the quenching substance they contained by means of a graph so that the expected background count for each of the control samples of the second series could be read and substracted from the activity counted for each of these samples to give the actual cpm for each of these samples. The ratio of the actual cpm to the known amount of activity added to each of the samples (the counting efficiency) was then correlated with the AES ratio obtained for each of these samples. The plot of the AES ratio versus counting efficiency could then be used to determine the counting efficiency of the experimental samples by correlation to their AES ratio.

The other method for determining counting efficiencies could be used with any type of experimental sample and was especially useful with

samples of unusual or unknown chemistry. This method consisted of adding a known amount of activity to the scintillation solution after the cpm value for the sample had been determined. The sample was then counted again and the counting efficiency could be calculated as the ratio of the gain in activity after addition of the standard to the amount of standard activity added.

Techniques for the Hydrolysis of Samples of Viomycin and Separation of the Hydrolysis Components by Ion Exchange Chromatography

Hydrolyses of both radioactively labelled and unlabelled viomycin were routinely carried out as follows: the sample of viomycin was dissolved in 6 N hydrochloric acid (35 ml per gram of viomycin), placed under a reflux condensor and heated at steam bath temperature for twenty hours. Samples of ^{14}C-labelled viomycin were normally diluted with two grams of commercial viomycin (Parke, Davis, and Company) that had been dried to a constant weight in a vacuum desiccator at room temperature. As samples of labelled viomycin were being hydrolyzed, nitrogen was slowly bubbled through the hydrolysis system and into a trap containing ten milliliters of a one molar solution of the hydroxide of Hyamine 10-X (Reg. Trademark of Rohme & Haas, Inc.) in methanol which was connected to the reflux condenser. The carbon dioxide released during hydrolysis was thus trapped as the carbonate salt of Hyamine hydroxide *(36)*.

After the hydrolysis was completed, the hydrolysate was concentrated to a volume of approximately ten milliliters and then stirred with IR-45(OH^-) resin (approximately ten milliliters of resin per gram of viomycin hydrolyzed) for an hour or until the pH had risen to about five.

The solution was filtered and the resin was washed with five or six resin volumes of water. The combined filtrate and washings were concentrated to a volume of about ten milliliters and the sample was then ready to be put onto the column.

The column used to chromatograph the viomycin hydrolysate was made from 24 mm (i.d.) glass tubing in two eight-foot sections joined by a ground glass ball and socket joint and equipped with a Teflon stopcock at one end. The Dowex 50 (H^+) resin (Dowex 50 W X-8, 100-200 mesh, Baker reagent 1930) used to pack the column was prepared initially by washing 1500 ml of wet resin with four one-liter portions of 4 N hydrochloric acid; each time the resin was allowed to settle and the supernatent liquid and fines were removed by decantation. The resin was then washed with one-liter portions of distilled water to a pH of five. The column was packed by slurrying the resin with 1200 ml of water; the slurry was then poured into the top of the column with the stopcock open so that the excess water drained off. Once all the resin had been added and allowed to settle for about five hours, the column was ready for use. The dimensions of the packed resin were 2.4 cm x 450 cm.

After each run of a hydrolysate over the column, the column was unpacked and the resin was regenerated by washing it with four one-liter portions of 5 N hydrochloric acid in a 150 mm (3 liter) Buchner funnel so that the liquid could be quickly removed by filtration after each washing. The resin was then washed with one liter portions of distilled water to a pH of five. The resin was then repacked in the column.

The hydrolysate sample was pipetted onto the top of the column and washed onto the resin with a small amount of water. The column was eluted

by gradient elution chromatography with hydrochloric acid. The two-liter erlenmeyer flasks were connected in series to the top of the column such that once flow through the column was begun, the hydrochloric acid in the first flask would siphon into the second flask as the solution in the second flask (which was stirred continuously using a magnetic stirrer to assure complete mixing) siphoned onto the column. The second flask was filled initially with distilled water while the first flask was filled initially with two liters of a hydrochloric acid solution made up by diluting 300 ml of concentrated hydrochloric acid to one liter. After the first two liters of hydrochloric acid had been added to the second flask, the first flask was kept filled with a solution made up by diluting 400 ml of concentrated hydrochloric acid to one liter.

Gradient elution using this type of apparatus follows Equation 1.

$$\ln \left(\frac{X}{X - c}\right) = \frac{v}{V} \qquad (1)$$

X = concentration of solution in the first flask

c = instantaneous concentration of the solution in the second flask. (Thus c is also equal to the instantaneous concentration of the solution being added to the column)

v = instantaneous volume of solution that has been added from the first flask to the second flask

V = volume of solution in the second flask (the mixing flask)

An automatic fraction collector (GM Instrument Company, Inc. Model VE-2002-B24) having a capacity of 237 18 x 150 mm test tubes was used to collect fractions by means of a constant volume attachment.

Twenty milliliter fractions were collected at a flow rate adjusted to approximately one milliliter per minute. A total of 400 fractions were normally collected.

Although the column was designed to separate the hydrolysate resulting from the hydrolysis of five grams of viomycin, samples ranging from one gram to twelve grams of viomycin were hydrolyzed, and the hydrolysate was separated into the various components without any marked change in resolution.

A five gram sample of viomycin was hydrolyzed, and the hydrolysate was prepared and chromatographed as described above. Each 20 ml fraction was added to a tared 50 ml erlenmeyer flask and lyopholized. In this way a complete weight curve was determined for the separation of the hydrolysate into the individual components. A plot of the weight of each fraction versus the fraction number is shown in Figure 2, and a summary of the data on the major fractions is given in Table 2. Identification of the component of each major peak was accomplished by TLC analysis.

Samples of DL-serine and L-diamonopropionic acid monohydrochloride were weighed and dissolved in 20 ml of 4.0 N hydrochloric acid. These solutions were lyopholized in the same manner as the fractions from the column and the increase in weight was determined. The weight of the serine sample was increased by 36 per cent, and the weight of the diaminopropionic acid sample was increased by 37 per cent.

Location of the radioactive fractions from the hydrolysate of radioactively labelled viomycin was accomplished by first determining the activities of every fourth fraction collected. Radioactive peaks

Table 2. Summary of the Principal Fractions from the Gradient Elution Chromatography of the Hydrolysate of Viomycin.

Fractions	Principal Component[a]	Weight[b] (mg)
20-25	Neutral Material	415
126-133	Serine	1818
162-168	Ammonium Chloride	430
229-240	Diaminopropionic Acid	1290
243-257	β-Lysine	1747
264-289	Viomycidine	764
346-357	Mixture of Peptides	165
358-379	Mixture of Peptides	351

[a]Principal components were identified by TLC.

[b]Sum of weights of lyopholized fractions.

were thus located, and the activity of every fraction containing a significant amount of racioactivity was then determined. The scintillation samples for the determination of the activity of these fractions were made up by diluting a 250 µl aliquot of the fraction being counted with 750 µl of water in a counting vial. Fifteen milliliters of tT21 scintillation solution was then added, and the mixture was shaken and allowed to stand until a clear solution resulted. The samples were counted as was described above. A quenching curve was made up for scintillation samples of this type using hydrochloric acid as the expected quenching

substance. A plot of counting efficiency versus AES ratio for scintillation samples containing one milliliter of hydrochloric acid solutions of varying concentration and fifteen milliliters of tT21 scintillation solution is given in Figure 1. It was found that a counting efficiency of 82 per cent could be used for the range of hydrochloric acid concentrations found in the fractions of interest noting that the aliquot added to the scintillation solution was diluted to one fourth of the original concentration in making up the sample.

The total activity (dpm) of each 20 ml fraction was calculated from equation 2,

$$(\text{dpm}) = (\text{cpm}) \times \frac{80}{0.82} \qquad (2)$$

where (cpm) refers to the activity counted for the sample in counts per minute.

Fractions containing significant amounts of the same hydrolysate component were pooled; the pooled sample was evaporated to dryness and redissolved in ten milliliters of water; this solution was lyopholized. These samples were saved for purification, counting, and degradation studies to determine their complete ^{14}C-labelling pattern.

Viomycin from Radioactively Labelled Precursors

Preparation and Purification

Samples of viomycin investigated were produced from an isolate of *Streptomyces griseus* var. *purpurea* at the University of Illinois under the direction of Dr. Paul D. Shaw. Production of viomycin was achieved

by growing the organism in one liter flasks containing 250 ml of a culture medium containing glucose, soybean meal, and inorganic salts on a rotary shaker at 150 rpm. Viomycin could be detected in the brew on the third day after inoculation. The concentration of viomycin increased for the next seven days and then remained constant for at least three additional days. Radioactively labelled compounds were added to the culture medium on the third day after inoculation; the cultures were harvested on the tenth day after inoculation *(37)*.

The viomycin produced was absorbed from the culture brew onto an IRC-50(Na^+) ion-exchange resin column. The viomycin was eluted from the column with 0.5 N hydrochloric acid solution after first washing the column with distilled water. The viomycin was then treated batchwise with Dowex-2 (OH^-) resin to pH 8, and the resulting solution was passed over a Dowex 2 ($SO_4^=$) column to convert the viomycin to the sulfate salt.

After the effluent was concentrated to approximately six milliliters, this solution of viomycin sulfate was chromatographed on a column (2 x 172 cm) of Sephadex G-15 using 0.01 M formic acid as the eluent. The effluent contained viomycin of about 80 per cent purity, but after rechromatography on a second Sephadex G-15 column, the viomycin obtained upon lyopholization of the effluent was pure as ascertained by biological and spectrophotometric assays and by TLC. The overall recovery of viomycin based on the effluent from the IRC-50 (Na^+) column was 75 per cent *(37)*.

Eight radioactive compounds suspected of being precursors of viomycin were added to the culture medium in separate experiments and the viomycin thus produced was isolated and purified. The eight compounds and their respective run numbers are as follows:

Run Number	Radioactive Precursor
I	Glucose-U-^{14}C
II	DL-Serine-3-^{14}C
III	DL-Serine-1-^{14}C
IV	DL-Lysine-2-^{14}C
V	DL-Glutamic-3,4-^{14}C Acid
VI	DL-Glutamic-5-^{14}C Acid
VII	DL-Arginine-5-^{14}C
VIII	DL-Aspartic-4-^{14}C Acid

Table 3 summarizes the data obtained concerning the samples of viomycin produced using these eight radioactive compounds as precursors.

Hydrolysis and Chromatography

The eight samples of radioactively labelled viomycin produced from the radioactive compounds suspected of being precursors of viomycin were hydrolyzed and chromatographed over the Dowex 50 (H^+) column as is described above. The weights of unlabelled and radioactivity labelled viomycin that were hydrolyzed for each run are given in Table 4.

The methanolic solution of the hydroxide of Hyamine 10-X used to trap the carbon dioxide released during the hydrolysis of the radioactively labelled viomycin samples was diluted to 50.00 ml with methanol. A 500 µl aliquot of each of these solutions was measured into a scintillation vial, 15 ml of the toluene scintillation solution was added, and

Table 3. Data Relating to the Production and Purification of Labelled Viomycin from Radioactive Precursors[a].

Precursor	Precursor Added (dpm)	Viomycin Produced Crude (mg)	Viomycin Carrier Added (mg)	Viomycin Isolated (mg)[b]	Viomycin Isolated (dpm)	% Incorporation[c]
Glucose-U-^{14}C	8.08×10^8	266	0	190(163)	7.45×10^6	1.51
Serine-3-^{14}C	9.53×10^8	184	68	201(170)	8.85×10^6	1.38
Serine-1-^{14}C	2.68×10^8	122	80	143(123)	4.87×10^6	3.13
Lysine-2-^{14}C	2.08×10^9	83	120	207(156)	2.73×10^7	1.91
Glutamic-3,4-^{14}C Acid	8.07×10^8	176	0	135(130)	2.04×10^6	0.32
Glutamic-5-^{14}C Acid	9.75×10^8	172	0	151(114)	1.13×10^6	0.175
Arginine-5-^{14}C	1.08×10^9	192	0	140(128)	2.94×10^7	4.08
Aspartic-4-^{14}C Acid	7.95×10^8	274	0	169(155)	8.37×10^5	0.186

[a]Paul D. Shaw, private communication.

[b]The value in parentheses represents the corrected weight of viomycin isolated based on spectrophotometric assay of final product.

[c]Based on activity of viomycin isolated corrected for losses during purification.

Table 4. Amounts of Labelled and Unlabelled Viomycin Hydrolyzed for each Run.

Run No.	Precursor	Amount of Viomycin Hydrolyzed[a] Labelled	Unlabelled	Total
I	D-Glucose-U-^{14}C	175	1126	1301
II	DL-Serine-3-^{14}C	186	2356	2542
III	DL-Serine-1-^{14}C	127	2218	2345
IV	DL-Lysine-2-^{14}C	181	2366	2547
V	DL-Glutamic-3,4-^{14}C Acid	113	2473	2586
VI	DL-Glutamic-5-^{14}C Acid	130	2582	2712
VII	DL-Arginine-5-^{14}C	112	2464	2576
VIII	DL-Aspartic-4-^{14}C Acid	146	2447	2593

[a]Expressed in milligrams as the sulfate salt dried to a constant weight at 25°C.

the sample counted and the counting efficiency determined as usual. The total activity of the carbon dioxide from the hydrolysis of a sample of viomycin was calculated from formula 3.

$$T.A. = \frac{cpm}{0.500} \times \frac{50}{E} = \frac{cpm}{E} \times 100 \qquad (3)$$

where T.A. = total activity of CO_2 produced, dpm.

cpm = activity counted for 500 µl aliquot

E = counting efficiency

These results are given in Table 5.

Table 5. Summary of the Activities of the Principal Fractions from the Hydrolysates of Radioactively Labelled Viomycin

Precursor	Fractions	Sample Identification	Sample Weight (mg)	Total Activity (dpm)
Glucose-U-^{14}C	142-148	Serine	598	1,570,000
(Run No. I)	174-177	--	15	42,000
	222-229	--	73	171,000
	250-260	DAP	250	1,210,000
	268-280	β-Lysine	338	507,000
	288-312	Viomycidine	219	244,000
	376-393	Peptides	84	171,000
	-	Carbon Dioxide	-	502,000
Serine-3-^{14}C	164-172	Serine	785	3,410,000
(Run No. II)	198-201	--	22	54,900
	277-290	DAP	486	816,000
	298-312	β-Lysine	626	430,000
	320-334	Viomycidine	111	78,200
	335-348	Viomycidine	229	105,000
	428-443	Peptides	111	118,000
	-	Carbon Dioxide	-	106,000
Serine-1-^{14}C	140-146	Serine	850	2,100,000
(Run No. III)	249-259	DAP	526	413,000
	270-276	β-Lysine	453	42,400
	-	Carbon Dioxide	-	580,000

Table 5. Summary of the Activities of the Principal Fractions from the Hydrolysates of Radioactively Labelled Viomycin (Continued)

Precursor	Fractions	Sample Identification	Sample Weight (mg)	Total Activity (dpm)
Lysine-2-^{14}C	135-142	Serine	1085	3,150,000
(Run No. III)	162-168	--	25	67,000
	212-230	--	41	160,000
	236-246	DAP	484	744,000
	249-266	β-Lysine	776	9,780,000
	267-279	--	109	1,760,000
	361-388	Peptides	210	200,000
	-	Carbon Dioxide	-	23,900
Glutamic-3,4-^{14}C Acid	142-148	Serine	869	101,000
(Run No. V)	246-254	DAP	456	125,000
	262-274	β-Lysine	658	366,000
	281-307	Viomycidine	358	216,000
	365-390	Peptides	194	101,000
	-	Carbon Dioxide	-	76,700
Glutamic-5-^{14}C Acid	133-139	Serine	993	99,100
(Run No. VI)	206-213	--	112	90,400
	232-239	DAP	461	46,200
	247-256	β-Lysine	546	99,100
	265-290	Viomycidine	365	105,000
	-	Carbon Dioxide	-	106,000

Table 5. Summary of the Activities of the Principal Fractions from the Hydrolysates of Radioactively Labelled Viomycin (Continued).

Precursor	Fractions	Sample Identification	Sample Weight (mg)	Total Activity (dpm)
Arginine-5-^{14}C	136-142	Serine	964	50,400
	211-220	--	116	181,000
	238-250	DAP	490	241,000
	251-260	β-Lysine	589	181,000
	268-279	Viomycidine	107	1,770,000
	280-288	Viomycidine	90	1,580,000
	289-301	Viomycidine	240	4,650,000
	305-317	--	48	763,000
	328-342	--	27	687,000
	350-368	Peptides	101	1,260,000
	369-379	Peptides	90	1,200,000
	380-386	Peptides	43	560,000
	387-393	Peptides	30	275,000
	394-404	Peptides	43	322,000
	-	Carbon Dioxide	-	63,800
Aspartic-4-^{14}C Acid	132-138	Serine	968	43,300
	211-216	--	106	51,700
	238-243	DAP	383	35,600
	254-264	β-Lysine	628	152,000
	-	Carbon Dioxide	-	75,200

Plots of radioactivities of the fractions obtained from the chromatography of the hydrolysates of these viomycin samples are shown in Figures 3 through 10. A summary of the identification and activities of the principal fractions from these separations is given in Table 5.

Purification and Determination of the Specific Activity of the Hydrolysis Components.

Serine. The lyopholized sample of serine resulting from the pooling of the fractions containing serine from each run was dissolved in a small amount of water and then stirred with fifteen to twenty milliliters of IR-45(OH^-) resin. The resin was filtered and washed, and the combined filtrate and washings were evaporated to dryness. The white solid was then dissolved in a small volume of distilled water. This solution was heated to boiling and ethanol was added until a slight cloudiness appeared; the solution was then allowed to cool to room temperature. The sample was then allowed to stand in a refrigerator overnight after which the white crystals were filtered, washed with ethanol and dried.

Approximately fifty milligrams of each sample of serine was weighed out exactly and dissolved in a small amount of water, and then this solution was diluted to a volume of 5.00 ml. A drop of chloroform was added to each solution to prevent the growth of mold. A 100 µl aliquot of each of these samples and fifteen milliliters of the tT21 scintillation solution was added to one milliliter of water in a scintillation vial and the mixture was shaken until a clear solution was

obtained. The activities of these samples were then counted as usual and the specific activities were calculated from formula 4. The results are given in Table 6.

$$S.A. = \frac{(cpm) x\ c}{E} \times 10 \qquad (4)$$

where S.A. = specific activity, dpm/mg

cpm = activity of sample

E = counting efficiency of sample

c = concentration of sample, g/ml.

Table 6. Activities of Purified Serine and Diaminopropionic Acid (DAP) Derived from Radioactively Labelled Viomycin

Precursor	Specific Activity (dpm/mg) Serine	DAP
Glucose-U-^{14}C	6480	6148
Serine-3-^{14}C	7020	2105
Serine-1-^{14}C	4580	1340
Lysine-2-^{14}C	6130	2009
Glutamic-3,4-^{14}C Acid	355	402
Glutamic-5-^{14}C Acid	206	134
Arginine-5-^{14}C	105	89
Aspartic-4-^{14}C Acid	82	103

The remainder of each of the serine solutions was saved to be used in the degradation reactions described below.

Diaminopropionic Acid. The lyopholized samples of diaminopropionic acid were crystallized by dissolving the samples in a small amount of boiling water and adding ethanol until cloudiness appeared. After water was added dropwise until the cloudiness disappeared, the sample was allowed to cool to room temperature whereupon crystals of diaminopropionic acid appeared. After standing in the refrigerator overnight, the sample was filtered and the crystals of diaminopropionic acid were washed with ethanol and then dried.

Approximately fifty milligrams of each sample of diaminopropionic acid was weighed out exactly and dissolved in a small amount of water, and then this solution was diluted to a volume of 5.00 ml. To prevent mold from growing, one drop of chloroform was added to each of the solutions. A 100 µl aliquot of each of these samples was added to one milliliter of water in a scintillation solution and the mixture was shaken until a clear solution was obtained. The activities of these samples were then counted as usual and the specific activities were calculated from formula 4. The results are given in Table 6.

The remainder of each of the DAP solutions was saved to be used in the degradation reactions described below.

β-Lysine. The lyopholized samples of β-lysine were converted to the p-hydroxyazobenzene-p'-sulfonate (PHABS) salts by adding dropwise with stirring to each sample dissolved in a small amount of water a saturated solution of PHABS until further addition of the solution gave no further precipitation. The mixture was then centrifuged and the

supernatent liquid pipetted off. The precipitate was washed with a small amount of water, and the mixture was centrifuged again. After pipetting the supernatent liquid, the precipitate was recrystallized from hot water. The crystals of the PHABS salt of β-lysine were collected by filtration and were washed with water and then dried. The activities of the PHABS salts of the β-lysine samples were not counted due to the insolubility of the salts.

Determination of the ^{14}C-Labelling Patterns of the Hydrolysis Components Serine and Diaminopropionic Acid

The C-3 carbon: reaction with periodate. The solutions of each of the samples of radioactive serine and diaminopropionic acid used in the determination of the specific activities were also used in these degradation reactions. For each sample, 100 µl of the solution of the amino acid and 4.5 ml of 0.5 M acetic acid-sodium acetate buffer of pH 4.7 were added to a 25 ml erlenmeyer flask. To this solution was added 2.4 ml of a freshly prepared solution of 0.5 M sodium meta-periodate (107 mg/ml), and the mixture was allowed to stand at room temperature for two and one-half hours. After this time, two milliliters of an ethanolic solution of dimedone (80 mg/ml of ethanol) was added, and the dimedone derivative of formaldehyde was allowed to crystallize for twenty hours. The crystalline precipitate was then filtered, washed with a small amount of water, and then recrystallized from ethanol-water. The recrystallized derivative was filtered, washed with water, and dried. In calculations, one milligram of the dimedone derivative of formaldehyde is equivalent to 0.3596 mg of serine or 0.4811 mg of

diaminopropionic acid monohydrochloride. In typical runs, the yield of unrecrystallized derivative was 99 per cent from serine and 92 per cent from diaminopropionic acid.

The radioactive dimedone derivatives were counted by weighing out to the nearest one-hundredth of a milligram approximately ten milligrams of the crystalline derivative in a scintillation vial, adding 15 ml of the toluene scintillation solution, and counting the activity and determining the counting efficiency as usual. The results of these degradations for the serine and diaminopropionic acid samples are given in Table 7.

Table 7. Labelling Patterns of Serine and Diaminopropionic Acid (DAP) Derived from Radioactively Labelled Viomycin

Precursor	Compound	Specific Activity (dpm/mg)	Activity at C-3 Position by Periodate[a]	Activity at C-1 Position by NBS[a]	Activity at C-1 Position by Ninhydrin[a]
Glucose-U-^{14}C	Serine	6480	2220	2100	2130
(Run No. I)	DAP	6148	2190	----	2110
Serine-3-^{14}C	Serine	7020	6460	517	573
(Run No. II)	DAP	2105	1890	----	206
Serine-1-^{14}C	Serine	4580	31	4560	4430
(Run No. III)	DAP	1340	13	----	1290
Lysine-2-^{14}C	Serine	6130	2130	146	148
(Run No. IV)	DAP	2010	741	----	43

[a]dpm per mg of serine or diaminopropionic acid reacted.

The C-1 carbon: reaction with N-bromosuccinimide. The samples of serine and diaminopropionic acid were reacted with N-bromosuccinimide in order to obtain carbon dioxide quantitatively from the C-1 carbon by a modification of the method of Chappelle and Luck *(38)*. The reaction flasks were constructed from 25 ml erlenmeyer flasks by sealing a 12 mm length of 8 mm (i.d.) pyrex tubing to the center of the bottom of the flasks to serve as a receptacle for a small vial made by sealing one end of a 20 mm length of 8 mm (o.d.) pyrex tubing. The small vial was supported in the glass receptacle by a piece of 8 mm (o.d.) tubing 12 mm long placed into the receptacle first. During the reactions the reaction flask was kept sealed with a rubber serum cap which also served to allow injection of solutions into either the reaction compartment or the small vial after the flask had been sealed.

The reaction of the serine samples was carried out by adding together in the reaction compartment of the reaction flask three milliliters of a ten per cent solution of succinimide in 1 M sodium acetate-acetic acid buffer solution at pH 4.7 and an aliquot of the serine sample containing approximately five micromoles of serine (50.0 µl of the solutions used to determine the specific activity of the serine samples). The flask was capped with the rubber serum cap and the reaction mixture was allowed to equilibrate for twenty minutes. At that time the flask was uncapped and 0.5 ml of N-bromosuccinimide reagent (made up by adding 2.5 g each of N-bromosuccinimide and succinimide to 25 ml of 1 M sodium acetate-acetic acid buffer solution at pH 4.7) was added to the reaction compartment. The flask was then tightly capped immediately, and 0.5 ml of a 1 M solution of the hydroxide of Hyamine in methanol was then injected into the small

vial. The reaction flask was placed in a horizontal shaker and shaken gently. After three hours of shaking the reaction and absorption of carbon dioxide was complete; the flask was uncapped, and the small vial was placed *in toto* into a scintillation vial. Fifteen milliliters of toluene scintillation solution was added, and the sample was thoroughly mixed before counting was begun. Some degree of care was necessary to obtain the correct count for these samples. Initially the count was much higher than the correct count, but these extraneous counts decayed within five or six minutes to the correct value. After such a sample had been standing for two to three hours, however, the count rose over a period of several hours to a value much higher than the correct count. Thus the procedure used to obtain the correct count was to measure a series of one minute counts until the initial extraneous counts had decayed and the count became constant. Then several ten minute counts could be taken to get an average value for the counts per minute for the sample. It was found that the counting efficiency of these samples was 85.0 ± 0.3 per cent, and so a value of 85.0 per cent was used for all samples of this type. Background for these samples was taken as 38.0 cpm. The results for the activities at the C-1 position of the serine samples are given in Table 7.

In order to test how completely the C-1 is converted to carbon dioxide, a 50 µl aliquot of a known sample of DL-serine-1-^{14}C (activity = 8080 dpm/ml) was reacted as described. The sample analyzed for 8090 dpm/ml at the C-1 position, or 100.0 per cent.

When the samples of diaminopropionic acid were reacted in exactly the manner described for the serine samples, anomalous results were

obtained in that for several samples more activity was indicated for the C-1 position than was possible after subtracting the activity present in the C-3 position from the total activity of the sample. In no case, however, did the activity exceed the total activity of the serine sample. In one case, that of diaminopropionic acid from DL-serine-1-^{14}C (Run No. III), 99.9 per cent of the total activity was indicated for the C-1 position.

The C-1 carbon: reaction with ninhydrin. The samples of serine and diaminopropionic acid were reacted with ninhydrin by a modification of the manometric method of Van Slyke and co-workers. The same reaction flasks as were used in the N-bromosuccinimide reaction were used in this reaction. A 50 µl aliquot of the sample of serine or diaminopropionic acid was added to three milliliters of a 1 M citric acid buffer solution of pH 2.5 in the reaction compartment of the reaction flask. Fifty milligrams of ninhydrin was added to this solution with a spatula and then an empty vial was put into place in the receptacle and the flask was tightly stoppered with a rubber serum cap. The reaction flask was then allowed to stand in a boiling water bath for five minutes for serine samples and ten minutes for diaminopropionic acid samples and was then removed and placed in an ice bath for several minutes. As soon as the reaction mixture had become cool, 0.5 ml of the 1 M solution of the hydroxide of Hyamine in methanol was injected into the vial and the reaction flask was shaken for three hours. The small vial was then removed and placed in toto in a scintillation vial. Fifteen milliliters of toluene scintillation solution was added and the sample was counted as was described for the reaction with N-bromo-

succinimide. Both diaminopropionic acid and serine reacted smoothly with ninhydrin under these conditions and the results obtained for the activities of the C-1 position of the samples of serine and diaminopropionic acid are given in Table 7.

β-Lysine

The methyl ester of di(N-phthalyl)-β-lysine. Several fractions containing β-lysine from previous hydrolyses using non-labelled viomycin were pooled and lyopholized to give 1.320 g of a brownish glass. To this glass was added 3.00 g of powdered phthalic anhydride and 1.5 g of powdered anhydrous sodium acetate. This mixture was ground together with a glass rod and then was heated in an oil bath at 170° for thirty minutes. The reaction mixture was then cooled, and 50 ml of 1 N hydrochloric acid and 50 ml of ether were added. After the mixture was stirred for two hours, all of the solid material had dissolved. The ether layer was separated, and the ether was removed by heating on a steam bath to yield a colorless oil. This oil was extracted five times with 20 ml portions of boiling water in order to remove the phthalic acid present. The solid that remained was dissolved in one liter of ether, and an ethereal solution of diazomethane was added until a yellow color persisted. The reaction mixture was then allowed to stand uncovered for three days whereupon clusters of white crystals were deposited. These crystals were collected and dried to yield 1.320 g of the methyl ester of di(N-phthalyl)-β-lysine, m.p. 156.5-157.5° [lit. *(9)* m.p. 155.5-156.0°].

A total of 628 mg of the PHABS salt of β-lysine from the hydrolysate of viomycin from lysine-2-^{14}C as precursor was stirred with 65 ml

of IR-45(OH^-) resin for an hour. The resin was removed by filtration, and the filtrate was concentrated to a volume of ten milliliters. This solution was lyopholized to yield 172 mg of a brown glass. This glass was mixed with 250 mg of anhydrous sodium acetate and 625 mg of powdered phthalic anhydride, and the methyl ester of di(N-phthalyl)-β-lysine was prepared as before. The yield was 191 mg of white crystals, which were recrystallized from methanol and which had a melting point of 154-155°. A portion (3.660 mg) of the methyl ester of di(N-phthalyl)-β-lysine having lysine-2-^{14}C as precursor was weighed out in a scintillation vial and dissolved in 15 ml of toluene scintillator solution. The activity and counting efficiency of this sample were determined as usual to give a total activity for the sample of 35,600 dpm. This corresponds to a specific activity of the β-lysine it was derived from (as the free base) of 28,000 dpm/mg.

Barbier-Wieland degradation of the methyl ester of di(N-phthalyl)-β-lysine. A total of 842 mg (2.0 mmoles) of the methyl ester of di(N-phthalyl)-β-lysine (made up by diluting 100 mg of the ester derived from the labelled β-lysine with 742 mg of the ester from unlabelled β-lysine) were dissolved in 60 ml of dry benzene. This solution was added to phenylmagnesium bromide prepared from 420 mg of magnesium (18 mmoles), 3.20 g (19 mmoles) of freshly distilled bromobenzene, and 40 ml of dry ether at 0° with stirring. The mixture was then boiled under reflux for 3.75 hr. Then, after cooling to 0°, the complex was decomposed by adding 15 ml of saturated ammonium chloride solution at 0°. The organic layer was separated from the aqueous

layer, and the aqueous layer was extracted twice with 20 ml portions of benzene. The combined organic extracts were evaporated to yield a brown oil. This oil was dissolved in twelve milliliters of acetic anhydride, and the resultant solution was boiled under reflux for 2.5 hr to effect dehydration of the alcohol. The acetic anhydride and acetic acid were removed by distillation under vacuum, and the oil that was obtained was dissolved in 70 ml of methylene chloride. The methylene chloride solution was cooled to -70° and a mixture of ozone and oxygen was passed through the solution until a blue color persisted. The solution was then allowed to warm to room temperature under vacuum. The oil that resulted was dissolved in 112 ml of glacial acetic acid, and to the resulting solution was added a mixture of 5.0 ml of water, 16 ml of 30 per cent hydrogen peroxide, and five drops of concentrated hydrochloric acid. The resulting mixture was allowed to stand at room temperature overnight. The solvent was then removed by distillation under vacuum, and the oil that resulted was mixed with 20 ml of water and was then extracted with 20 ml of ether. The aqueous layer and the portion of the oil that had not gone into solution were then extracted with 30 ml of benzene. The ether and benzene extracts were combined and extracted with 50 ml of a 10 per cent sodium carbonate solution. The organic layer was evaporated to yield a colorless oil. This oil was extracted with 30 ml of ligroin and the ligroin solution was concentrated to about five milliliters. The concentrated ligroin extract was passed over an alumina column (1.0 cm x 14 cm) using benzene-ligroin (1:1) as the eluent. The fifth fraction upon evaporation deposited 14.4 mg (4.0%,

based on methyl ester of di(N-phthalyl)-β-lysine) of benzophenone, m.p. 47-48.0° [lit. *(40)*, m.p. 48°]. The sample resulting from dissolving 14.40 mg of this benzophenone in 15 ml of toluene scintillation showed an activity of 326 dpm.

Upon acidification of the combined sodium carbonate extracts with five per cent hydrochloric acid, a white precipitate formed. This precipitate was collected by filtration and recrystallized from 95% ethanol to yield 70 mg (8.9% yield, based on methyl ester of di(N-phthalyl)-β-lysine) of L-di(N-phthalyl)ornithine, m.p. 183-186° [lit. *(9)* m.p. 187-188.5°].

Ornithine: reaction with ninhydrin. To 68.0 mg of L-di(N-phthalyl)ornithine isolated from the Barbier-Wieland degradation was added 324 mg prepared from unlabelled L-ornithine by the method of van Tamelen and Smissman *(9)*. The total sample (392 mg, 1.00 mmole) was dissolved in 2.0 ml of 1 M alcoholic hydrazine hydrate and 2.0 ml of 95 per cent ethanol, and the solution was refluxed for 1.5 hr. The resulting suspension was evaporated to dryness on the steam-bath, and the solid residue was mixed with 20 ml of five per cent hydrochloric acid. After being heated for 15 min at 50° and slowly cooled to room temperature, the mixture was filtered. The filtrate was concentrated to approximately three milliliters, and then as it was being heated ethanol was added dropwise until the first crystals began to appear. After cooling the mixture for several hours in the refrigerator, the crystals were collected, washed with ethanol, and dried to give 80 mg (47.5%) of ornithine hydrochloride having an infrared spectrum (pellet) identical to that of authentic L-ornithine monohydrochloride.

A solution containing 37.3 mg of the ornithine monohydrochloride in 5.00 ml of water was prepared. A 100 µl aliquot of this solution was diluted to 1.0 ml and was added to fifteen milliliters of tT21 scintillation solution. The activity of this sample was determined to be 388 dpm.

A 100 µl aliquot of this same solution was reacted with ninhydrin exactly as was described for the serine and diaminopropionic acid samples earlier. The activity of the absorbed carbon dioxide was determined in the usual manner to be 315 dpm.

Attempted Determination of the Absolute Configuration of Viomycidine

Acetylviomycidine was prepared using the method of Miller *(16)*. The recrystallized product had mp 252-254° dec. and $[\alpha]_D^{28°} = +42° \pm 2°$ (c 1.0, water) [lit. *(16)*, mp 256-257° dec., $[\alpha]_D^{28°} = 41° \pm 1°$ (c 2.4, water)]. The n.m.r. spectrum of the sample was identical with the n.m.r. spectrum obtained by Miller.

The sample of acetylviomycidine was dissolved in 100 ml of 80 per cent formic acid solution; ozonolysis, oxidation, hydrolysis, and purification was carried out exactly as was described by Nasser *(11)* for the ozonolysis of a basic preparation of acetylviomycidine.

A small amount of material was obtained that gave a faint spot on TLC corresponding to aspartic acid, but the sample could not be recrystallized and it gave an infrared spectrum different from the infrared spectrum of either DL-aspartic acid or L-aspartic acid.

Attempted Syntheses of 2-Guanidino-Δ^1-pyrroline-5-carboxylic Acid

Attempts at the Reductive Cyclization of Ethyl 4-Cyano-2-nitrobutyrate

Ethyl 4-Cyano-2-nitrobutyrate. Ethyl 4-cyano-2-nitrobutyrate was prepared as described elsewhere *(41)*. The oily product was distilled in vacuo, and the fraction boiling at 117-120° (0.7 mm) [lit. *(41)*, bp 148-151° (4 mm)] was collected (80% yield).

The infrared spectrum showed absorptions at λ_{max} 3.34, 4.44, 5.72, 6.40, 6.97, 9.16 and 11.67 μ, among others. The n.m.r. spectrum (neat, TMS external) showed absorptions at τ 4.86 (1H, triplet, J = 7.0), 5.92 (2H, quartet, J = 7.1), 7.51-7.73 (4H, multiplet), and 8.92 (3H, triplet, J = 7.1.

Catalytic Hydrogenation of Ethyl 4-Cyano-2-nitrobutyrate. A solution of ethyl 4-cyano-2-nitrobutyrate (2.07 g, 11.1 mmoles) in 20 ml of methanol was added to 2.5 g of Raney nickel. This mixture was hydrogenated at 20° and one atmosphere pressure until absorption of hydrogen ceased. A total of 42.9 mmoles of hydrogen was absorbed (3.9 moles of hydrogen per mole of ethyl 4-cyano-2-nitrobutyrate). After the reaction was complete, the mixture was filtered through celite and a sintered glass funnel. The filtrate was evaporated to yield a dark intractable oil which gave a complex thin-layer chromatogram and ill-defined infrared and n.m.r. spectra.

Reduction of Ethyl 4-Cyano-2-nitrobutyrate with Zinc and Acetic Acid. A solution of ethyl 4-cyano-2-nitrobutyrate (1.86 g, 10.0 mmoles) in 25 ml of glacial acetic acid and 0.5 ml of water was cooled to 13° with a cold-water bath. As this solution was being stirred at 13°, 3.0 g of zinc dust was added at a rate of 0.5 g per half-hour. After the

addition of the zinc dust was complete, the mixture was stirred for an additional two hours at 13°. The reaction mixture was then filtered, and the residue was washed with cold glacial acetic acid. The filtrate and washings were combined and allowed to stand overnight. The small amount of zinc salts that crystallized upon standing was filtered, and the acetic acid was removed from the solution by distillation in vacuo. A brown oil remained that gave ill-defined infrared and n.m.r. spectra. Chromatography of this oil over an AG-1 (OH^-) ion-exchange column (85 ml of resin) yielded a small amount of a zinc salt when the column was eluted with water. Upon elution of the column with 1 N hydrochloric acid, a small amount of an oil was obtained that darkened quickly upon standing and gave ill-defined n.m.r. and infrared spectra.

Attempted Synthesis via Pyrrolidone-5-Carboxylic Acid

2-Methoxy-Δ^1-pyrroline. 2-Methoxy-Δ^1-pyrroline was prepared by a modification of the method of Peterson and Tietze *(42)*. Over a period of two hours 184 g (1.46 moles) of redistilled dimethyl sulfate was added to a solution of 2-pyrrolidone (124 g, 1.46 moles) in 500 ml of benzene at reflux. The solution was then allowed to reflux for sixteen hours. At the end of this time, the dark solution was cooled to 5°, and an excess of 50 per cent potassium carbonate solution was added. After evolution of carbon dioxide had ceased, the solution tested basic with litmus paper. The benzene layer was separated and washed twice with 25 ml portions of cold water. The benzene solution was dried over magnesium sulfate and then distilled. The following four fractions were collected: fraction 1, bp range 115-121° (740 mm Hg); fraction 2, bp 121° (740 mm

Hg); fraction 3, bp range 121-130° (740 mm Hg); and fraction 4, bp range 135-142° (15 mm Hg). A large amount of tar remained in the distillation pot after the distillation was complete. Nuclear magnetic resonance analysis of these four fractions indicated that fraction 1 contained benzene and 2-methoxy-Δ^1-pyrroline (n.m.r. spectrum (neat, TMS) showed absorptions at τ 6.37 (singlet, 3H), 6.40-6.75 (complex multiplet, 2H), and 7.48-8.47 (complex multiplet, 4H). Fraction 2 contained 2-methoxy-Δ^1-pyrroline and a small amount of benzene. Fraction 3 contained 2-methoxy-Δ^1-pyrroline and N-methyl-2-pyrrolidone (n.m.r. spectrum (neat, TMS) showed a complex band from 6.17-8.53τ (6H) and a singlet at 7.32 (3H)). Fraction 4 contained only N-methyl-2-pyrrolidone. Fractions 1 and 3 were combined and redistilled to yield a fraction boiling at 110-116° (740 mm Hg) containing 2-methoxy-Δ^1-pyrroline and a small amount of benzene as indicated by n.m.r. analysis. This fraction together with fraction 2 gave a total of 18.8 g (13%) of a clear liquid containing about 95 per cent 2-methoxy-Δ^1-pyrroline [lit. *(42)*, bp 118-121°, 760 mm Hg]. Further purification was not attempted.

2-Amino-Δ^1-pyrroline Hydrochloride. To a mixture of ammonium chloride (5.35 g, 0.1 mole) and 50 ml of absolute ethanol 2-methoxy-Δ^1-pyrroline (9.9 g, 0.1 mole) was added. This mixture was allowed to stand at room temperature for 31 hr. At the end of this time, the white crystalline material was filtered from the solution, and the filtrate was evaporated to yield a white solid. This white solid was recrystallized from acetonitrile and dried to yield 3.60 g (30%) of white crystals of the hydrochloride salt of 2-amino-Δ^1-pyrroline with mp 168-170° [lit. *(43)*, mp 169-171°].

The n.m.r. spectrum (18%, D_2O, DSS) showed absorptions at τ 6.37 (triplet, J = 6.9, 2H), 7.15 (triplet, J = 7.2, 2H), and 7.83 (quintet with additional fine splitting, 2H). The n.m.r. spectrum in TFA showed, in addition to the same absorptions shown in D_2O shifted to slightly lower τ values, absorptions at τ 2.17 (broad singlet, 1H) and 2.75 (broad singlet, 2H).

Attempted Preparation of 2-Guanidino-Δ^1-pyrroline

From 2-methoxy-Δ^1-pyrroline. To a solution of 4.95 (0.05 mole) of 2-methoxy-Δ^1-pyrroline in 30 ml of absolute ethanol was added 4.78 g (0.05 mole) of guanidine hydrochloride. After standing at room temperature for three days, the solution was filtered and distilled in vacuo, which yielded an orange oil that could not be crystallized. Inspection of the n.m.r. spectrum in TFA showed only one kind of proton attached to nitrogen. The oil also gave a negative Sakaguchi test.

From 2-amino-Δ^1-pyrroline hydrochloride. Aminoguanidine nitrate was prepared from aminoguanidine bicarbonate as described elsewhere *(11)*. From 10.0 g (74.0 mmoles) of aminoguanidine bicarbonate, 8.51 g (85.1%) of aminoguanidine nitrate was obtained, mp 145-145.5° [lit. *(11)*, mp 144-145°; lit. *(44)*, mp 144°].

1-Guanyl-3,5-dimethylpyrazole nitrate was prepared as described elsewhere *(45)*. From 5.00 g (50.0 mmoles) of 2,4-pentanedione and 6.80 g (50.0 mmoles) of aminoguanidine nitrate 6.85 g (80.5%) of white needles of 1-guanyl-3,5-dimethylpyrazole nitrate was obtained, mp 167-168° [lit. *(45)*, mp 166-168°].

To a solution of 2.010 g (10 mmoles) of 1-guanyl-3,5-dimethylpyrazole nitrate in 15 ml of water 1.205 g (10.0 mmole) of 2-amino-Δ^1-

pyrroline hydrochloride was added. After refluxing this solution overnight, the cooled reaction mixture was extracted three times with ten milliliter portions of diethyl ether. The ether extracts were combined and evaporated to dryness to give 96 mg of 3,5-dimethylpyrazole (10%). The aqueous layer was evaporated to dryness to give a white solid that gave a negative Sakaguchi test.

L-2-Pyrrolidone-5-carboxylic Acid. L-2-Pyrrolidone-5-carboxylic acid was prepared by a slight modification of the method of Hardegger and Ott *(46)*. Solid L-glutamic acid (125.0 g, 0.635 mole) was heated with stirring at 155-160° in an oil bath. After heating for an hour or more a melt began to form. Gradually all the solid melted, whereupon the melt was heated for an additional two hours. Upon cooling, the melt crystallized and was dissolved in 125 ml of hot water. Upon standing overnight at room temperature 14.60 g (13.4%) of DL-2-pyrrolidone-5-carboxylic acid crystallized from this solution, mp 181-182° [lit. *(46)*, mp 184°], which was filtered and dried. The filtrate was concentrated to approximately half its original volume and placed in the refrigerator overnight, whereupon L-2-pyrrolidone-5-carboxylic acid crystallized. The crystals were filtered and dried to yield 57.3 g of L-2-pyrrolidone-5-carboxylic acid, mp 162-163° [lit. *(46)*, mp, 164-165°]. The filtrate was again concentrated to half volume and an additional 15.2 g of L-2-pyrrolidone-5-carboxylic acid was obtained. The total yield of recrystallized L-2-pyrrolidone-5-carboxylic acid was 72.5 g (65.0%).

Methyl 2-Pyrrolidone-5-carboxylate. Methyl-2-pyrrolidone-5-carboxylate was prepared in best yield by dissolving the solid pyrrolidone-5-carboxylic acid prepared from 125 g of L-glutamic acid, without

recrystallization, in one liter of redistilled methanol. Dry hydrogen chloride was bubbled into the solution for several minutes, and the solution was then boiled under reflux overnight. Water was removed by adding 70 ml of benzene and distilling 150 ml of azeotrope. More dry hydrogen chloride was bubbled into the solution and the solution was boiled under reflux overnight for a second time. Water was removed azeotropically once more and the procedure was repeated a third time. Following the third azeotropic removal of water, excess methanol was removed by distillation. The resulting brown oil was distilled under vacuum to yield 58.0 g (47.7%) of methyl 2-pyrrolidone-5-carboxylate as a light yellow oil, bp 141-142° (1.0 mm Hg) [lit. *(47)*, bp 180° (12 mm)]. The solid that remained in the distillation flask was retreated with methanol and hydrogen chloride as described above. Distillation of the resulting brown oil yielded an additional 44.5 g of methyl pyrrolidone-5-carboxylate (total yield, 84.5%).

The n.m.r. spectrum (20% in $CDCl_3$, TMS) showed absorption at τ 2.15 (broad singlet, 1H), 5.53-5.80 (unresolved multiplet, 1H), 6.23 (singlet, 3H), and 7.44-8.17 (complex multiplet, 4H).

Attempted Preparations of Methyl 2-Methoxy-Δ^1-pyrroline-5-carboxylate.

Reaction of methyl 2-pyrrolidone-5-carboxylate with dimethyl sulfate. Dimethyl sulfate (25.2 g, 0.2 mole) was added dropwise with stirring at 60° to a solution of methyl 2-pyrrolidone-5-carboxylate (28.3 g, 0.2 mmole) in 70 ml of benzene. This mixture was allowed to boil under

reflux for 20 hr. and was then cooled to 5° in an ice bath. The mixture was extracted with 150 ml of 50 per cent potassium carbonate solution and was then washed with cold water. The benzene layer was separated and dried. After evaporation of the benzene, the brown oil was distilled to yield 10.2 g (35.7%) of methyl N-methylpyrrolidone-5-carboxylate, bp 150-156° (6-8 mm Hg) [lit. *(46)*, bp 75° (high vacuum)]. None of the desired methyl 2-methoxy-Δ^1-pyrroline-5-carboxylate was obtained.

The n.m.r. spectrum (neat, TMS) showed singlets at τ 6.18 (3H) and at 7.19 (3H) and complex multiplets at 4.40-5.87τ (1H) and at 7.44-8.13τ (4H).

Reaction of methyl 2-pyrrolidone-5-carboxylate with methyl iodide and silver oxide. Methyl 2-pyrrolidone-5-carboxylate (7.30 g, 0.05 mole) was dissolved in 30 ml of acetonitrile, and 10 g of freshly prepared silver oxide was added with stirring. With the stirring continuing, 15 ml of methyl iodide was slowly added. The mixture was then filtered, and the filtrate was evaporated to half of its original volume. An n.m.r. spectrum was taken of this solution. This spectrum showed the presence of methyl N-methylpyrrolidone-5-carboxylate (by the presence of the N-methyl group) and the presence of unreacted starting material (by the presence of N-H absorption). A peak present in the O-methyl region appeared to be split, indicating the possible presence of an O-methyl group other than the methyl ester, which would indicate the desired product might have been formed. In order to calculate the amount of the desired product that might have been formed, the following peaks were expanded and

the area under these determined with a planimeter: the peak at 2.16τ due to the presence of a proton bound to nitrogen represented unreacted starting material and had a relative area of 118; the complex absorption at 5.5-5.8τ was due to the proton on the carbon containing the carboxylate group represented the total amounts of the compounds of interest and had a relative area of 496; and the peak at 7.2 due to the N-methyl protons represented the amount of N-methylated product and had a relative area of 863/3 = 288. The reaction mixture thus contained 118/496 x 100 or 24% unreacted started material, 288/496 x 100 or 58% of N-methylated product, and thus by difference only a maximum of 18% of the desired methyl 2-methoxy-Δ^1-pyrroline-5-carboxylate could have been present. Upon distillation of the reaction mixture only acetonitrile, methyl iodide, methyl 2-pyrrolidone-5-carboxylate, and methyl N-methyl-pyrrolidone-5-carboxylate were obtained.

2-Amino-Δ^1-Pyrroline-5-carboxamide Hydrochloride. Triethyloxonium fluoborate was prepared as described elsewhere *(48)*. From 28.4 g (0.200 mole) of freshly distilled boron trifluoride etherate and 14.0 g (0.151 mole) of freshly distilled epichlorohydin was obtained 24.3 g (84.8%) of triethyloxonium fluoborate.

To a solution of methyl 2-pyrrolidone-5-carboxylate (18.2 g, 0.128 mole) in 100 ml of methylene chloride triethyloxonium fluoroborate (24.3 g, 0.128 mole) was added with stirring. The mixture was allowed to stir for two hours, and then the solvent was evaporated to yield a brownish oil. The n.m.r. spectrum of a small amount of this oil (50%, CH_2Cl_2, TMS) showed absorptions at τ 0.18 (broad singlet, 1H), 5.34 (quartet, J = 7.0, 2H), 6.19 (singlet, 3H), and 8.50 (triplet, J = 7.0, 3H) as well as

complex multiplets at 5.03-5.28τ (1H) and 6.62-7.71τ (4H).

The oil was dissolved in 400 ml of absolute ethanol saturated with ammonia, and this solution was allowed to stand at room temperature for two days. The crystalline material in the reaction mixture was filtered, the filtrate was concentrated to half of its original volume, and the crystalline material that resulted was again filtered. The filtrate was evaporated to yield a brown oil. Trituration of this oil with two 20-ml portions of cold methylene chloride yielded a white gum. This gum was dissolved in 50 ml of water, and this solution was passed over an IR-45 (Cl^-) column (800 ml of resin washed to pH 2.5). The column was eluted with water and ten 125-ml fractions were collected. Fractions 3-5 yielded white crystalline material upon evaporation. These three fractions were combined to give 18.7 g (81.7% based on methyl 2-pyrrolidone-5-carboxylate) of 2-amino-Δ^1-pyrroline-5-carboxamide hydrochloride. After recrystallization from ethanol-water the product showed mp 225-227° and $[\alpha]_D^{20°}$ = 0.00 ± 0.02°.

The infrared spectrum (pellet) of the compound showed λ_{max} 3.20, 3.40, 5.93, 6.08, 7.08, 7.72, 9.16, 11.03 , among others. The n.m.r. spectrum (22%, D_2O, DSS) showed an absorption at 5.32τ (6H) and a complex band of absorptions at 6.80-8.11τ (4H). The n.m.r. spectrum in TFA (15%) showed absorptions at τ1.83 (broad singlet, 1H), 2.13-3.00 (unresolved multiplet, 4H), 5.33-5.70 (triplet, J = 7.3, 1H), and 7.07-8.33 (complex multiplet, 4H).

A small amount of the compound was recrystallized twice from ethanol-water for elemental analysis. The analytical sample showed mp 227-229°.

Anal. $C_5H_{10}N_3OCl$ Calc'd: C, 36.70; H, 6.16; N, 25.69; Cl, 21.67 (163.61) Found: C, 36.89; H, 6.25, N, 25.49, Cl, 21.63.

Attempted Preparation of 2-Guanidino-Δ^1-pyrroline-5-carboxamide.

A solution of 4.02 g (10 mmole) of 1-guanyl-3,5-dimethylpyrazole nitrate in 200 ml of 1 N sodium hydroxide solution was extracted four times with 20 ml portions of ethyl acetate. The ethyl acetate extracts were combined and dried briefly over magnesium sulfate. After filtration, the solvent was removed under vacuum to yield a clear oil. This oil was dissolved in 5.2 ml of water, and 720 mg (2.37 mmole) of 2-amino-Δ^1-pyrroline-5-carboxamide hydrochloride was added. Two milliliters of triethylamine was also added and the solution was boiled under reflux for three hours. The still warm solution was extracted twice with 20 ml portions of ethyl acetate. The aqueous layer was distilled *in vacuo* to yield a clear oil. Trituration of this oil with several portions of ethyl acetate caused the oil to solidify. The solid was recrystallized from ethanol-water to yield 705 mg of crystals, mp 221-227°. The n.m.r. spectrum of the material (TFA) was identical to the n.m.r. spectrum of the starting material, 2-amino-Δ^1-pyrroline-5-carboxamide hydrochloride, except for the presence of peaks indicating a small amount of triethylamine hydrochloride.

Collection and Reduction of X-ray Diffraction Data for Viomycidine Hydrobromide

Twelve grams of commercially available viomycin sulfate was hydrolyzed in 6.0 N hydrochloric acid for twenty hours on the steam bath, and the hydrolysate was neutralized and chromatographed over a Dowex-50(H^+)

column using gradient elution as described earlier. Four hundred fractions (20 ml each) were collected, and the location of the viomycidine fractions was estimated from the weight curve shown in Figure 2. Beginning with fractions 251 and continuing through fraction 320, fractions were pooled in groups of five. The pooled fractions were evaporated to dryness *in vacuo*, redissolved in ten milliliters of water, and lyopholized. A crude weight curve was obtained which showed two peaks corresponding to the two peaks in the viomycidine fractions in Figure 2. The center fractions of the second peak, pooled fractions 291-295 (0.292 g lyopholized weight), was neutralized with IR-45(OH^-) resin and then converted to the stoichiometric hydrobromide salt by passage over an IR-45(Br^-) column containing 50 ml of resin washed to a pH of 3.0. Evaporation of the effluent yielded a solid which was crystallized from ethanol-water to yield 147 mg of white crystals of viomycidine hydrobromide, mp 205-212° (decomp).

Recrystallization of a portion of the viomycidine hydrobromide from methanol-water yielded crystals from which a crystal (approximately 0.2 mm x 0.1 mm x 0.1 mm) was selected and mounted on a standard goniometer head and affixed on a Buerger precession camera. After alignment of the crystal, Mo-K_α radiation was employed to obtain diffraction data from the *hkx* and *hxl* levels, where x = 0.3*. Three intensity exposures of 50.0, 5.0 and 0.5 hr duration were recorded for each of the levels.

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*For a complete discussion of X-ray diffraction camera techniques see M. J. Buerger, "X-ray Crystallography", John Wiley and Sons, Inc., New York, 1942. A discussion of the precession camera techniques is found in M.J. Buerger, "The Precession Method", John Wiley and Sons, New York, 1964.

The crystal was orthorhombic and all exposures showed the systematic absence $\underline{h} + \underline{k} = 2\underline{n}$, which indicated a C-centered lattice. Systematic absences in the $00\underline{l}$ levels for $\underline{l} = 2\underline{n}$ indicated as 2_1 axis along the $\underline{c}$ direction. A C-centered lattice with a 2_1 axis in the $\underline{c}$ direction occurs uniquely in the space group $C222_1$ *(49)*. (Viomycidine hydrochloride was also reported to be orthorhombic with space group $C222_1$ *(11)*.) Cell dimensions were $\underline{a} = 9.36 \pm 0.01$ Å, $\underline{b} = 12.47 \pm 0.01$ Å, and $\underline{c} = 15.29 \pm 0.01$ Å. Based on eight molecules per unit cell a density of 1.87 g/cc was calculated; an experimental density of 1.85 g/cc was obtained by the flotation method.

The intensities for each of the three exposures for each level were estimated visually using a standard intensity series, and these values were corrected for Lorentz-polarization effects as described previously *(50)*. A total of 397 unique non-zero reflections was obtained.

A Patterson synthesis which revealed a consistent set of vectors of high intensity was calculated as described previously *(50)*. The fractional coordinates of the bromide ion were obtained from the vector lengths and a structure factor *(51)* based on these bromide ion coordinates gave an R value of 0.30. An election density map *(52)* was calculated with phases of F_o based on the bromide ion. Eleven of the twelve remaining atoms could be located from this map. The value for the structure factor R fell to 0.115 with the inclusion of these eleven additional atoms. An election density map based on these twelve atoms enabled the location of the final atom. Upon varying isotropic temperature factors, $\underline{x}$, $\underline{y}$, and $\underline{z}$ coordinates of each of the thirteen atoms, and individual layer scale factors, the R value converged to a

final value of 0.090. Table 8 lists the observed and calculated structure factors for each h k l.

Table 9 lists the x, y, and z parameters and the isotropic temperature factors for each atom. The estimated limit of error is given in parentheses beside each value and corresponds to the uncertainty in the last significant digit. The scale factors were 0.0538, 0.0600, 0.0546, 0.0554, 0.0587, 0.0555, 0.0515, and 0.0554 for the h k 0, h k 1, h k 2, h k 3, h 0 l, h 1 l, h 2 l, and h 3 l layers respectively.

TABLE 8. CALCULATED AND OBSERVED STRUCTURE FACTORS FOR VIOMYCIDINE HBR

H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC
0	4	0	16	6	8	10	0	21	20	6	2	1	34	37	5	9	2	20	21
0	6	0	31	36	9	1	0	24	21	6	4	1	24	21	5	11	2	32	29
0	8	0	104	102	9	7	0	10	9	6	6	1	38	41	5	13	2	20	17
0	10	0	26	17	10	0	0	10	15	6	10	1	18	15	6	0	2	13	13
0	12	0	27	23	10	2	0	22	22	6	12	1	18	15	6	2	2	13	6
0	14	0	24	13	10	6	0	30	28	7	1	1	32	32	6	4	2	9	10
0	16	0	33	28	10	8	0	9	10	7	3	1	26	28	6	6	2	14	13
1	3	0	42	38	10	10	0	20	16	7	5	1	24	27	7	1	2	26	29
1	5	0	25	25	11	1	0	9	10	7	7	1	25	29	7	3	2	55	58
1	7	0	7	1	12	0	0	23	22	7	9	1	23	21	7	5	2	46	51
1	11	0	9	9	12	2	0	8	11	8	2	1	25	25	7	9	2	11	9
2	0	0	236	242	0	2	1	81	86	8	4	1	18	21	7	11	2	25	24
2	2	0	42	43	0	4	1	121	121	8	6	1	27	26	7	13	2	16	14
2	4	0	18	7	0	6	1	43	44	8	10	1	11	11	8	2	2	11	11
2	6	0	90	82	0	8	1	18	12	8	12	1	11	9	9	1	2	16	19
2	8	0	95	94	0	10	1	42	43	9	1	1	26	27	9	3	2	31	32
2	10	0	33	33	0	12	1	17	16	9	3	1	15	16	9	5	2	27	28
2	12	0	10	10	0	14	1	15	13	9	5	1	11	10	9	9	2	11	13
2	14	0	14	12	1	1	1	34	34	9	7	1	11	15	9	11	2	11	13
2	16	0	22	16	1	3	1	59	64	10	2	1	15	13	11	3	2	12	17
3	1	0	25	26	1	5	1	31	30	10	4	1	15	19	11	5	2	11	12
3	3	0	13	16	1	7	1	58	56	10	6	1	11	13	0	4	3	128	134
3	5	0	7	5	1	9	1	32	31	11	1	1	11	13	0	6	3	48	45
3	7	0	8	10	1	11	1	27	24	11	7	1	10	12	0	8	3	25	29
4	2	0	96	100	1	13	1	10	9	0	4	2	29	27	0	10	3	23	22
4	4	0	8	5	2	2	1	56	65	0	6	2	29	32	0	12	3	25	25
4	6	0	72	68	2	4	1	126	116	1	3	2	122	120	1	3	3	15	15
4	8	0	67	64	2	6	1	27	33	1	5	2	66	63	1	5	3	14	18
4	10	0	32	33	2	8	1	20	19	1	7	2	38	35	1	7	3	38	37
4	14	0	22	16	2	10	1	34	30	1	11	2	49	41	1	9	3	55	46
5	1	0	35	35	2	12	1	32	27	1	13	2	40	34	1	11	3	29	25
5	5	0	20	19	2	14	1	11	9	2	0	2	28	26	2	2	3	54	47
5	7	0	21	22	3	1	1	109	102	2	2	2	20	26	2	4	3	101	109
5	11	0	14	17	3	5	1	21	18	2	4	2	16	19	2	6	3	22	24
6	0	0	46	53	3	7	1	31	30	2	6	2	29	27	2	8	3	8	11
6	2	0	76	78	3	9	1	36	33	2	8	2	8	9	2	10	3	16	18
6	4	0	9	4	3	11	1	10	11	3	1	2	49	48	2	12	3	33	30
6	6	0	16	14	4	0	1	13	11	3	3	2	69	72	3	1	3	100	106
6	8	0	42	39	4	2	1	47	43	3	5	2	78	83	3	3	3	23	25
6	10	0	22	18	4	4	1	70	72	3	7	2	42	37	3	5	3	18	15
6	14	0	24	18	4	6	1	25	30	3	9	2	32	33	3	7	3	53	50
7	1	0	24	20	4	8	1	16	16	3	11	2	35	31	3	9	3	37	34
7	3	0	9	13	4	10	1	17	14	3	13	2	27	23	4	0	3	10	10
7	5	0	9	11	4	12	1	31	29	4	0	2	7	7	4	2	3	55	51
7	7	0	16	19	5	1	1	63	62	4	2	2	28	24	4	4	3	65	58
7	9	0	14	13	5	3	1	8	15	4	4	2	32	33	4	6	3	27	26
8	0	0	28	28	5	5	1	19	24	5	1	2	46	47	4	10	3	14	15
8	2	0	38	38	5	7	1	32	35	5	3	2	66	68	4	12	3	29	26
8	6	0	21	23	5	9	1	34	35	5	5	2	75	74	5	1	3	58	59
8	8	0	29	28	6	0	1	9	17	5	7	2	9	12	5	3	3	38	38

TABLE 8. (CONTINUED)

H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC	H	K	L	FO	FC
5	5	3	38	39	4	0	13	21	13	7	1	9	18	20	6	2	12	24	25
5	7	3	48	45	4	0	14	14	10	7	1	11	23	28	6	2	13	11	14
5	9	3	33	34	4	0	15	10	7	7	1	13	11	12	8	2	4	11	17
6	2	3	28	29	4	0	16	26	22	7	1	15	15	15	8	2	5	15	19
6	4	3	29	26	6	0	4	61	74	9	1	5	21	25	8	2	8	11	10
6	6	3	35	31	6	0	5	21	19	9	1	7	26	30	8	2	9	11	17
6	10	3	22	20	6	0	8	50	54	9	1	11	11	20	10	2	4	12	16
6	12	3	12	10	6	0	12	22	18	0	2	4	124	109	1	3	5	43	33
7	1	3	35	38	6	0	13	10	3	0	2	5	157	151	1	3	6	58	55
7	3	3	31	32	6	0	14	10	14	0	2	7	62	63	1	3	7	25	23
7	5	3	47	43	6	0	16	14	14	0	2	8	90	95	1	3	8	20	17
7	7	3	33	28	8	0	4	38	40	0	2	9	64	70	1	3	9	8	11
7	9	3	23	23	8	0	8	36	35	0	2	11	30	32	1	3	10	39	42
8	0	3	19	17	8	0	12	17	16	0	2	12	31	29	1	3	11	29	26
8	2	3	24	24	8	0	16	8	11	0	2	13	28	26	1	3	12	9	11
8	6	3	30	24	10	0	4	10	17	0	2	14	24	22	1	3	13	14	16
8	10	3	12	15	10	0	8	19	19	0	2	16	15	18	1	3	14	29	29
9	1	3	30	32	10	0	12	16	13	0	2	17	16	16	3	3	4	21	18
9	3	3	12	15	1	1	4	56	49	0	2	18	11	11	3	3	5	23	20
9	5	3	12	13	1	1	5	48	43	2	2	4	81	86	3	3	6	75	69
9	7	3	12	15	1	1	6	39	33	2	2	5	77	81	3	3	7	42	45
9	9	3	12	12	1	1	7	56	52	2	2	6	7	10	3	3	8	14	14
10	2	3	17	16	1	1	9	34	36	2	2	7	55	54	3	3	9	17	20
11	1	3	12	14	1	1	10	30	28	2	2	8	68	66	3	3	10	49	47
0	0	16	34	27	3	1	6	40	43	2	2	16	19	18	5	3	6	75	72
0	0	14	30	26	3	1	5	71	63	2	2	14	10	15	5	3	5	9	9
0	0	12	67	62	3	1	4	43	37	2	2	13	31	29	5	3	4	17	18
0	0	10	66	63	1	1	15	30	29	2	2	12	28	26	3	3	14	26	28
0	0	8	62	59	1	1	14	10	14	2	2	11	24	25	3	3	13	10	11
0	0	6	42	39	1	1	13	13	17	2	2	10	8	10	3	3	12	20	16
0	0	4	206	203	1	1	11	43	43	2	2	9	44	47	3	3	11	25	27
2	0	4	101	106	3	1	7	69	67	2	2	17	19	20	5	3	7	25	25
2	0	5	9	8	3	1	8	30	28	4	2	4	64	60	5	3	8	13	19
2	0	6	9	14	3	1	9	20	19	4	2	5	32	30	5	3	9	10	14
2	0	7	35	32	3	1	10	35	34	4	2	6	12	15	5	3	10	38	39
2	0	8	61	64	3	1	11	50	53	4	2	7	24	25	5	3	12	21	22
2	0	9	39	37	3	1	15	36	32	4	2	8	30	36	5	3	14	23	22
2	0	10	34	29	5	1	4	32	33	4	2	9	22	24	7	3	5	15	17
2	0	11	8	10	5	1	5	47	45	4	2	10	16	17	7	3	6	43	54
2	0	12	56	60	5	1	6	30	32	4	2	11	14	10	7	3	7	11	10
2	0	13	26	23	5	1	7	40	48	4	2	12	26	28	7	3	8	15	14
2	0	14	16	13	5	1	8	18	20	4	2	13	23	25	7	3	9	11	13
2	0	16	33	28	5	1	9	21	23	4	2	16	11	14	7	3	10	30	31
4	0	4	57	59	5	1	10	25	27	4	2	17	12	15	7	3	12	12	13
4	0	5	28	26	5	1	11	37	39	6	2	4	33	34	7	3	14	17	17
4	0	6	8	5	5	1	13	10	12	6	2	5	27	27	9	3	6	29	29
4	0	7	18	18	5	1	15	26	23	6	2	6	14	13	9	3	10	21	24
4	0	8	63	66	7	1	5	26	32	6	2	8	17	22	0	0	0	0	0
4	0	9	12	14	7	1	6	10	12	6	2	9	23	25	0	0	0	0	0
4	0	12	41	39	7	1	7	32	39	6	2	10	15	19	0	0	0	0	0

Table 9. Atomic Position Parameters and Isotropic Temperature Factors for Viomycidine Hydrobromide.

Atom	x	y	z	B($Å^2$)
Br	0.2446 (4)	0.0631 (3)	0.1300 (2)	3.48 (7)
O(1)	0.334 (3)	0.176 (2)	0.552 (2)	3.9 (6)
O(2)	0.104 (2)	0.141 (2)	0.575 (2)	3.6 (5)
N(1)	0.298 (2)	0.320 (2)	0.433 (2)	2.3 (5)
N(2)	0.404 (3)	0.191 (2)	0.338 (2)	2.8 (5)
N(3)	0.194 (3)	0.085 (2)	0.375 (2)	3.0 (5)
N(4)	0.415 (2)	0.000 (2)	0.356 (2)	2.5 (4)
C(1)	0.203 (4)	0.186 (3)	0.536 (3)	3.0 (7)
C(2)	0.163 (4)	0.256 (3)	0.467 (3)	3.3 (8)
C(3)	0.118 (3)	0.186 (3)	0.379 (3)	2.6 (6)
C(4)	0.169 (4)	0.263 (3)	0.301 (2)	2.5 (7)
C(5)	0.326 (4)	0.292 (3)	0.335 (3)	3.7 (8)
C(6)	0.338 (3)	0.089 (3)	0.354 (2)	3.0 (7)

Note: The number in parentheses represente the estimated error in the last significant digit.

CHAPTER III

DISCUSSION OF RESULTS

The purpose of this research was to initiate a study of the biosynthesis of the antibiotic viomycin by Streptomyces griseus var. purpurea. Since viomycin is a peptide which yields upon acid hydrolysis the three unusual amino acids, L-α,β-diaminopropionic acid, L-β-lysine, and a guanidino amino acid called viomycidine, in addition to L-serine, carbon dioxide, ammonia, and urea, the major area of investigation of this research logically was the study of the biosynthesis of these unusual amino acids.

It was planned to include in the scope of this research a proof of the proposed structure of viomycidine either by synthesis or by other means. Knowledge of the structure of viomycidine would be necessary for a meaningful interpretation of any carbon-14 incorporation data obtained for viomycidine as well as for future planning of degradation reactions to enable the determination of labelling patterns in ^{14}C-labelled viomycidine.

Biosynthetic Studies

The proposed method of investigation of the biosynthesis of viomycin, and in particular of the unusual amino acid fragments of viomycin, was to produce viomycin in the presence of ^{14}C-labelled compounds suspected of being precursors of one or more of the fragments of

viomycin. The ^{14}C labelled viomycin was then to be hydrolyzed. The hydrolysis components were to be separated and the activities of each component determined. Further degradation was also planned for any of the hydrolysis components found to contain significant radioactivity in order to determine the complete labelling pattern for those compounds.

Methods of separation of the hydrolysis components of viomycin described in the literature *(4,10)* were found to be difficult and involved, and an investigation was undertaken to develop an efficient and reproducible method of separation. Dowex 50 ion-exchange resin has been frequently used to separate complex mixtures of amino acids *(53)*, and its applicability to the separation of the hydrolysis components of viomycin was studied. It was found that a column (2.4 cm x 450 cm) containing approximately 1400 ml of Dowex 50-W X8 (H^+) ion exchange resin when eluted with a concentration gradient of hydrochloric acid would achieve a complete separation of the hydrolysis components. It was also determined that the results obtained for the separation of the components were quite reproducible provided that 20 ml fractions were collected at a flow rate of approximately one milliliter per minute during each separation. In one run the sulfate salt equivalent of 5.0 g of the free base of viomycin was hydrolyzed for twenty hours in 6 N hydrochloric acid on a steam bath. After neutralization of the hydrolysate, it was chromatographed over the Dowex 50 column. A total of 380 fractions were collected; each fraction was lyopholized, and the lyopholized weight of the fraction was determined. A standard weight curve was thus obtained (Figure 2) that could be used to locate specific

components in later separations. In a later chromatography of the hydrolysate of twelve grams of viomycin, each hydrolysis component was located by reference to the standard weight curve. The fractions containing each component corresponded almost exactly to the fractions indicated by the weight curve.

The first major peak, fractions 21-27 (see Figure 2), contained all of the neutral and any acidic material present in the hydrolysate. This peak should actually contain only urea and column throw, but a very dark brown oil was obtained. A trace of urea was present as indicated by a positive test with p-dimethylaminobenzaldehyde reagent *(5)*, but nothing could be obtained crystalline from the oil. The inability to obtain anything more than a trace of urea in any of the separations carried out will be discussed in more detail later. The other major peaks are listed and identified in Table 2* The weights listed for each component are the sum of the weights of the lyopholized fractions containing that component and are thus not to be taken as precise measures of the actual weights of the components. In experiments to determine the gain in weight that occurs when samples are lyopholized from hydrochloric acid solution, it was determined that the weight of serine samples increased by 36 per cent and the weight of diaminopropionic acid samples increased by 37 per cent. If the weights of the components are converted to moles, the ratios of the moles of components are serine:diaminopropionic acid: β-lysine:viomycidine:ammonium

*p. 34, this thesis.

chloride:: 2.00:1.08:1.19:0.48:0.94 (arbitrarly taking serine as 2.00, with no corrections for excess weight for serine and diaminopropionic acid). All of the components of the hydrolysate yield solids upon lyopholization except β-lysine, which yields a glass that oils when it is exposed to the air. Thus the β-lysine fractions probably contain more water than the serine or diaminopropionic acid fractions. The ratio of β-lysine is thus somewhat larger than it probably should be. Even so, the ratios given above agree reasonably well with the accepted values for the occurrence of these residues in viomycin (2:1:1:1) *(8,12)*.

Eight radioactively labelled compounds were investigated with respect to their incorporation into viomycin. Uniformly labelled glucose was chosen for the first experiment because it was expected that significant labelling would appear in each of the hydrolysis components. This run could thus serve as a test of the experimental method of separating the hydrolysis components and counting the activity of the fractions in order to locate the radioactive fractions. Hydrolysis and chromatography were carried out exactly as had been done with samples of unlabelled viomycin, except that steps were taken to trap the carbon dioxide that was released. As the fractions were being collected, scintillation samples were prepared and counted for every fourth fraction. In this way a peak was located for every hydrolysis component except urea. Based on these results, the activities of the remaining fractions in each of the peaks were counted. The activities are plotted versus fraction number in Figure 3. The fractions containing the major hydrolysis components were easily identified by comparing Figure 3 with the weight curve shown in Figure 2.

A peak corresponding to urea was searched for by determining the activity of every second fraction in the region where urea should have appeared. No radioactivity was found in this region. Subsequently it was found that a radioactive peak corresponding to urea was observed in none of the experiments involving radioactive precursors.

For each fraction the activity counted (cpm) could be converted to the actual activity (dpm) of the fraction by application of equation 2*. The total activity for each component was calculated by summing up the activities counted (cpm) for all fractions containing a particular component and applying equation 2* to the sum. These values are shown in Table 5** and are discussed below.

The method just discussed for the experiment with glucose-U-^{14}C proved to be entirely satisfactory and was used in the seven remaining experiments involving labelled precursors.

It might be expected that each carbon atom in the viomycin produced with glucose-U-^{14}C as precursor would contain an equal amount of radioactivity. Thus each carbon atom might be expected to contain 1/23 of the total activity. Since the sample of viomycin hydrolyzed contained an activity of 6.82×10^6 dpm, each carbon atom would be expected to have an activity of 2.97×10^5 dpm. It is thus possible to estimate the total activity expected for each of the hydrolysis components. These values are compared with the experimentally observed

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*p. 34 , this thesis

**p. 40, this thesis

values in Table 10. It should be noted that soybean meal was available

Table 10. Comparison of Predicted and Observed Activities of the Hydrolysis Components of Viomycin from D-Glucose-U-^{14}C.

Compound	Total Activity (dpm) Predicted[a]	Observed[b]
Carbon Dioxide	297,000	502,000 (1.7)
Urea	297,000	----------[c]
Serine	1,780,000	1,570,000 (0.89)
Diaminopropionic Acid	891,000	1,210,000 (1.4)
β-Lysine	1,780,000	507,000 (0.28)
Viomycidine	1,780,000	244,000 (0.14)

[a]On the basis of an equal amount of activity in each carbon atom.

[b]The value in parentheses is the ratio of the observed activity to the predicted ativity.

[c]No urea was isolated

as a source of carbon in the growth medium of S. griseus in addition to the glucose-U-^{14}C that was added. It would be difficult to correct the predicted activities for this complication except by noting qualitatively that compounds close to glucose metabolically, such as carbon dioxide, might contain a relatively larger amount of activity than compounds that would require a large member of steps to be synthesized from glucose. This prediction is consistent with the results obtained except for

diaminopropionic acid. The diaminopropionic acid had roughly twice the activity per carbon atom that the serine had. This situation could arise if at least part of the serine to be incorporated into viomycin as a seryl residue had been bound to a cell wall prior to the addition of the labelled glucose, so that it would be available for incorporation as a serine residue but not available for conversion to diaminopropionic acid.

The sum of the activities of the major peaks plus the activity of the carbon dioxide from the viomycin from glucose-U-^{14}C was 4.42 x 10^6 dpm, or 65 per cent of the activity of the sample hydrolyzed. The remainder of the activity would probably be found associated with material remaining on the column or in the fractions not included in the major radioactive peaks.

In the next two experiments DL-serine-3-^{14}C and DL-serine-1-^{14}C were used as precursors. The plots of activities are given in Figures 4 and 5, and the principal fractions are summarized in Table 5*. As would be expected, in both cases the serine isolated from the hydrolysate contained the largest amount of activity of any of the hydrolysis products. In the case of DL-serine-3-^{14}C as precursor, 42 per cent of the total activity hydrolyzed appeared in the serine fractions; in the case of DL-serine-1-^{14}C as precursor, 48 per cent of the total activity hydrolyzed appeared in the serine fractions. Of even more significance is the fact that in both cases relatively high activities also appeared in the diaminopropionic acid fractions. The ratio of activity of diamino-

*p. 40, this thesis.

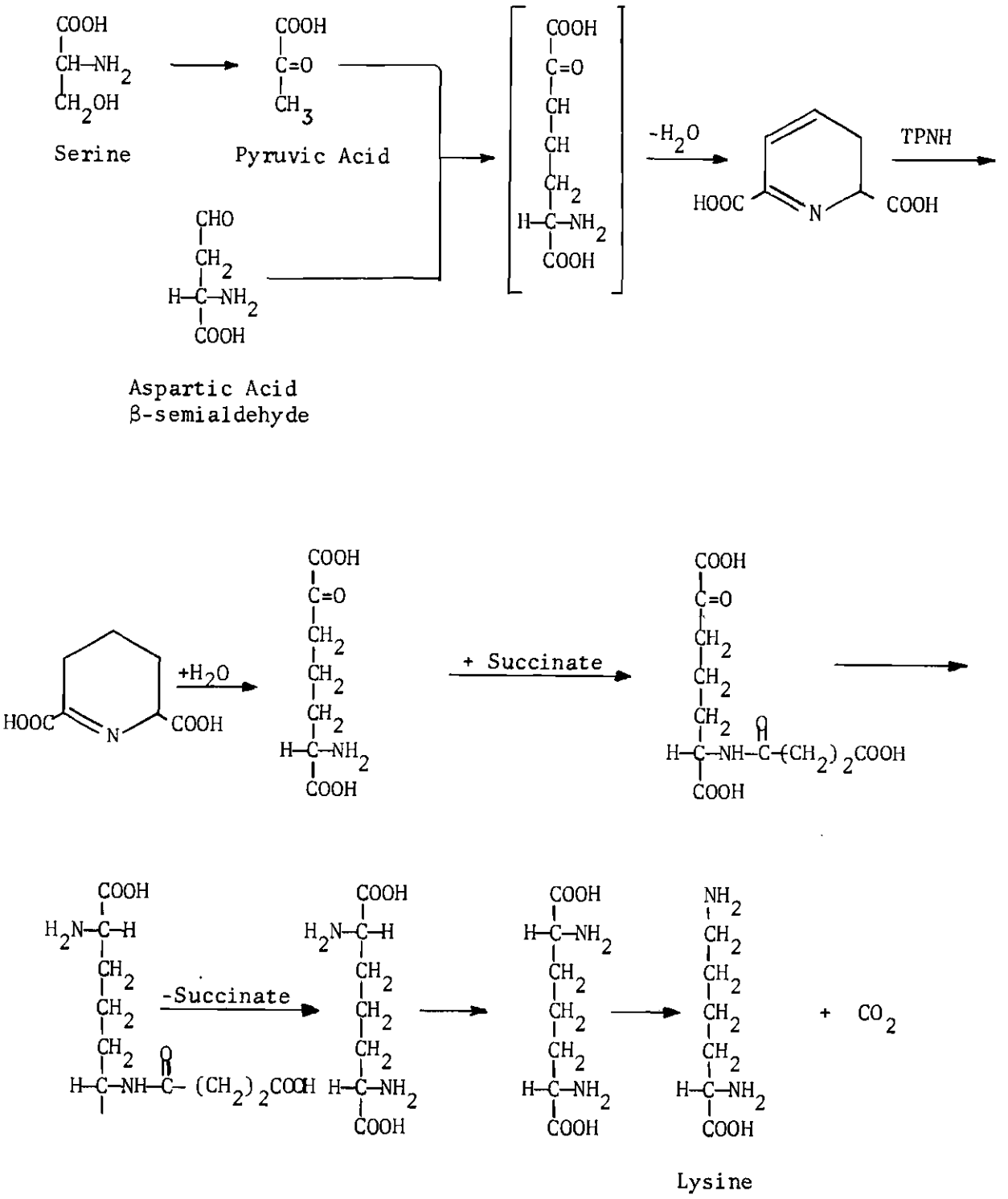
propionic acid to the activity of serine was 0.20 and 0.24 for DL-serine-3-^{14}C and DL-serine-1-^{14}C as precursors, respectively. These data suggest that serine might be the precursor of diaminopropionic acid in viomycin.

The fact that the β-lysine fractions contained a significant level of activity with DL-serine-3-^{14}C as precursor, but very little activity with DL-serine-1-^{14}C as precursor is consistent with at least one of the two known biosynthetic pathways to lysine *(54)*. This pathway, the diaminopimelic acid pathway shown in Scheme 1, is the one that might have been chosen a priori as the one operative in Streptomyces griseus since the Streptomyces are closely related to the bacteria in which the diaminopimelic acid pathway was first reported *(54)*. Furthermore, one strain of Streptomyces griseus has been reported to have the diaminopimelic acid pathway on the basis of incorporation of aspartic-4-^{14}C acid *(55)*.

In the diaminopimelic acid pathway L-lysine is produced via a condensation of pyruvate and aspartic acid β-semialdehyde. Since the degradation of serine to pyruvate is well established *(56)*, a pathway exists for the incorporation of serine in lysine. Since the C-1 carbon of pyruvate (which corresponds to the C-1 carbon of serine) is lost in the transformation of meso-α,ε-diaminopimelic acid (the final intermeciate in the pathway) to L-lysine, it would be expected that lysine from serine-3-^{14}C would be labelled. This explanation of course requires the assumption that lysine is the precursor of β-lysine.

The activity of the carbon dioxide from DL-serine-1-^{14}C was 5.5 times the activity of the carbon dioxide from DL-serine-3-^{14}C even though the viomycin from DL-serine-3-^{14}C was twice as radioactive as

Scheme 1


Serine
Pyruvic Acid
Aspartic Acid
β-semialdehyde
-H2O
TPNH
+H2O
+ Succinate
-Succinate
+ CO2
Lysine

the viomycin from DL-serine-1-^{14}C. This result is consistent with several of the common biological degradations serine undergoes: the conversion of a phosphatidylserine to a phosphatidylaminoethanol by decarboxylation or conversion to pyruvate and decarboxylation of the pyruvate *(57)*.

A total of 64 and 72 per cent of the activity of the viomycin hydrolyzed was recovered in the carbon dioxide and the major fractions of DL-serine-3-^{14}C and DL-serine-1-^{14}C as precursors, respectively.

Since there was evidence that β-lysine could be obtained from lysine in strains of Clostridium and Escherichia *(29, 31)*, the next experiment, using DL-lysine-2-^{14}C as precursor, was designed to determine if lysine is the precursor of β-lysine in viomycin.

The results, shown in Figure 6 and summarized in Table 5, clearly indicate that β-lysine can be derived from lysine. A total of 41 per cent of the activity of the viomycin hydrolyzed appeared in the β-lysine fractions. Unexpectedly, a peak appeared in this run which did not correspond to any of the known hydrolysis products of viomycin and which did not appear in any of the other experiments. This peak (see Figure 6) which appeared just after the β-lysine peak and just before the normal position for viomycidine contained a significant amount of radioactivity but had a lyopholyzed weight of only 109 mg. Attempts were made to characterize the active component of this peak, but the sample contained significant amounts of both β-lysine and viomycidine. It is possible that this peak corresponds to either 2,5-diaminohexanoic acid or 3,5-diaminohexanoic acid, both known metabolites of lysine *(31, 58)*. This would require that the "β-lysine isomer" was incorporated into a "viomycin-like" molecule since the sample of viomycin hydrolyzed had been chromatographed over two

Sephadex columns.

The serine and diaminopropionic acid fractions also contained relatively high activities. The ratio of the activity of the diamonopropionic acid fractions to the activity of the serine fractions was 0.24, the same value that was obtained from this ratio when DL-serine-1-^{14}C was used as precursor. A direct pathway from lysine to serine would seem to be indicated by the relatively high incorporation of activity in serine when DL-lysine-2-^{14}C was used as precursor. There are no reports of a biosynthetic pathway from lysine to serine in the literature; conjuctures as to possible pathways will be discussed later in conjunction with a discussion of the labelling pattern of the serine sample in question.

In the next two experiments DL-glutamic-3,4-^{14}C acid and DL-glutamic-5-^{14}C acid were tested as precursors. The results are shown in Figures 7 and 8 and are summarized in Table 5*. In both cases incorporation of activity into the viomycin molecule was low and none of the hydrolysis products showed significant activity.

The seventh labelled compound to be investigated was DL-arginine-5-^{14}C. The results for this experiment, shown in Figure 9 and summarized in Table 5*, are very interesting in that significant activity appeared only in the viomycidine fractions and in certain fractions eluted after viomycidine. These fractions must contain compounds more basic than viomycidine and thus it would appear that these compounds are peptides which contain the viomycidine residue. One of these compounds has been

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*p. 40, this thesis.

isolated, purified, and shown to yield viomycidine and diaminopropionic acid upon acid hydrosysis *(59)*.

The viomycidine fractions, which appeared to involve three overlapping peaks (see Figure 9), all gave the same two spots by TLC.

Several facts support the suggestion that arginine is the precursor of the viomycidine residue in viomycidine. The extremely high level of activity found in the viomydicine fractions for the arginine-5-^{14}C run compared to the low levels of activity found in viomycidine fractions from all other precursors investigated is significant. The next most active viomycidine sample, that from glucose-U-^{14}C, contained less than three per cent of the activity of the viomycidine from arginine-5-^{14}C. Also, arginine and viomycidine are both guanidino amino acids. Furthermore, the molecular formulas of viomycidine and arginine differ by only four hydrogen atoms.

In the final experiment in this series, DL-aspartic-4-^{14}C acid was used as precursor. As was the case with both labelled glutamic acid samples, a very low level of activity was incorporated into the viomycin grown using DL-aspartic-4-^{14}C acid (see Figure 10 and Table 5). None of the hydrolysis components thus showed a signficant level of activity. β-lysine was the most active of the hydrolysis components, as might have been expected if the diaminopimelic acid pathway were operative*, as was suggested earlier.

The labelling patterns of the samples of serine and diaminopropionic acid that showed significant ^{14}C-incorporation were determined

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*Scheme 1, p. 80 this thesis.

in order to demonstrate more conclusively that serine is the precursor of diaminopropionic acid in viomycin. The samples of serine and diaminopropionic acid from the precursors glucose-U-^{14}C, serine-1-^{14}C, serine-3-^{14}C and lysine-2-^{14}C were thus subjected to degradation reactions designed to determine the labelling patterns of these compounds.

Reaction of both serine and diaminopropionic acid with periodate would be expected to yield carbon dioxide from the C-1 carbon, formic acid from the C-2 carbon, and formaldehyde from the C-3 carbon *(60)*. There is in the literature a method for separately counting each of these degradation products *(61)*. This method involves separate oxidations of the formic acid and formaldehyde to carbon dioxide followed by trapping and counting the carbon dioxide samples. However, since the two oxidations were shown not to be quantitative, this procedure requires that the carbon dioxide must be converted to a carbonate salt that can be weighed and counted as such.

It was decided that the simplest procedure for determining the labelling patterns for the serine and diaminopropionic acid samples would involve two separate reactions. First, a periodate oxidation followed by formation, collection, and counting of the dimedone derivative of the formaldehyde produced would yield the activity present in the C-3 position. Second, there are two known methods for quantitatively converting the carboxyl group (C-1) of α-amino acids to carbon dioxide. The first involves reaction with ninhydrin *(39)*; the second involves oxidation by N-bromosuccinimide *(38)*. Collection, followed by counting of the carbon dioxide produced would yield the activity present in the C-1 position. The activity present in the C-2 position would be

determined by difference.

The reaction of serine and diaminopropionic acid with periodate presented no difficulty when carried out at pH 4.7 *(62)*. The formaldehyde was produced and converted to the dimedone derivative in yields up to 99 per cent for serine and 92 per cent for diaminopropionic acid. After recrystallizing and counting a known amount of the dimedone derivative, the activity present at the C-3 position per gram of serine or diaminopropionic acid monohydrochloride was calculated.

In order to test the procedure using known compounds, samples of the DL-serine-3-^{14}C and DL-serine-1-^{14}C that were used as precursors were reacted with periodate. The dimedone derivatives of the formaldehyde formed were prepared, recrystallized, and the activities were counted. The DL-serine-3-^{14}C sample was assayed for 91.8 per cent of the total activity at the C-3 psoition and the DL-serine-1-^{14}C sample assayed for 0.0 per cent of the total activity at the C-3 position. These data are included in Table 11.

Samples of serine and diaminopropionic acid having a significant activity (Runs I-IV) were also reacted with periodate. The results for the activities of these samples at the C-3 positon are given in Table 7.

The reaction of serine and diaminopropionic acid with N-bromosuccinimide was studied. The method of Chappelle and Luck was used *(38)* with slight modifications as to the reaction vessel and the method of collecting the carbon dioxide produced. This procedure was tested using samples of the DL-serine-3-^{14}C and DL-serine-1-^{14}C used as precursors. The results indicated that 7.8 and 99.9 per cent of the total activity of the DL-serine-3-^{14}C and DL-serine-1-^{14}C samples, respectively, were

Table 11. Labelling Patterns as Per Cent of Specific Activity of Precursor Samples of DL-Serine-1-^{14}C and DL-Serine-3-^{14}C and Labelling Patterns as Per Cent of Specific Activity of Serine and Diaminopropionic Acid (DAP) Samples Derived from Labelled Viomycin.

Sample	% Activity at C-1[a]	% Activity at C-2[b]	% Activity at C-3
Precursor Glucose-U-^{14}C			
Isolated Serine	32.6	33.1	34.3
Isolated DAP	34.4	31.0	34.6
Precursor Serine-1-^{14}C	99.9	0.1	0.0
Isolated Serine	98.2	1.1	0.7
Isolated DAP	96.3	2.7	1.0
Precursor Serine-3-^{14}C	7.8	0.4	91.8
Isolated Serine	7.8	0.3	91.9
Isolated DAP	9.8	0.5	89.7
Precursor Lysine-2-^{14}C			
Isolated Serine	2.4	62.9	34.7
Isolated DAP	2.1	61.0	36.9

[a]Average of NBS and ninhydrin runs for serine. The results for the C-1 position in the serine samples were identical within experimental error for the two methods.

[b]%C-2 = 100 - (%C-1 + %C-3).

present at the C-1 position. The results are consistent with the results obtained for the C-3 position and thus establish the labelling patterns of the two precursor serine samples. These data are shown in Table 7*.

When the significantly active diaminopropionic acid samples were reacted with the N-bromosuccinimide reagent, however, results for the C-1 position were obtained which were not consistent with the results obtained for the C-3 position of these compounds. In two cases the sum of the activities obtained for the C-1 and C-3 positions was greater than the total activity of the sample. It was concluded that over-oxidation of the diaminopropionic acid samples was occurring in the reaction with N-bromosuccinimide.

It was then decided to investigate the ninhydrin method of Van Slyke, et al. *(39)*. Van Slyke's method was followed with only modification in the reaction apparatus and method of collection of carbon dioxide as noted earlier. The four samples of serine that had been assayed with N-bromosuccinimide reagent were also reacted by the ninhydrin method. The results for the C-1 position which are given in Table 7 were identical within experimental error with the results by the N-bromosuccinimide method. The four samples of labelled diaminopropionic acid were then reacted by the ninhydrin method. The results for the C-1 position obtained by this method were consistent with the results for the C-3 position and are given in Table 7.

The per cent of the total activity present in the C-1 and C-3

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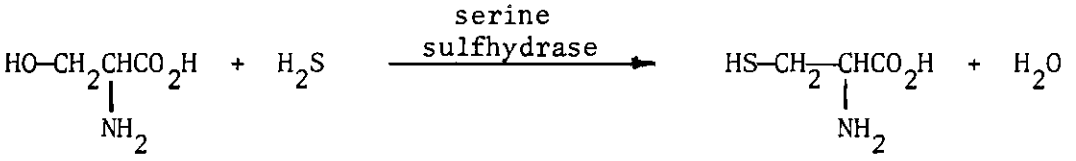
*p. 47, this thesis.

positions was calculated for each of the four serine and four diaminopropionic acid samples. The values for the C-2 position were calculated by difference. For the C-1 position of the serine samples an average of the ninhydrin and N-bromosuccinimide values was calculated. These values, as well as the corresponding values for the two precursor serine samples, are given in Table 11.

Examination of Table 11 and comparison of the values for serine and diaminopropionic acid having the same precursor reveals that the agreement of the labelling patterns between serine and diaminopropionic acid in each case is virtually identical. As an indication of the reliability of method and data, the values for serine and diaminopropionic acid from precursor glucose-U-^{14}C may be compared with the value of 33.3 per cent that would be predicted for each of the carbon atoms in both compounds. The labelling patterns for the run using serine-1-^{14}C as precursor are nearly identical; the values indicate that the three carbon atoms of the precursor serine were converted into those of serine in viomycin to an extent of 98 per cent and into those of diaminopropionic acid to an extent of 96 per cent. The values for the run using serine-3-^{14}C as precursor indicate an incorporation of the three carbon atoms as one unit of the precursor serine into the serine of viomycin to an extent of 100 per cent and into the diaminopropionic acid to an extent of 98 per cent. These results strongly suggest that serine (all three atoms as one unit) is the direct biosynthetic precursor of diaminopropionic acid. It is possible that aminomalonic acid semialdehyde is an intermediate in this conversion as was suggested by Reinbothe *(24)* but other intermediates may also be possible. Another possible mechanism would involve direct addition of ammonia to serine

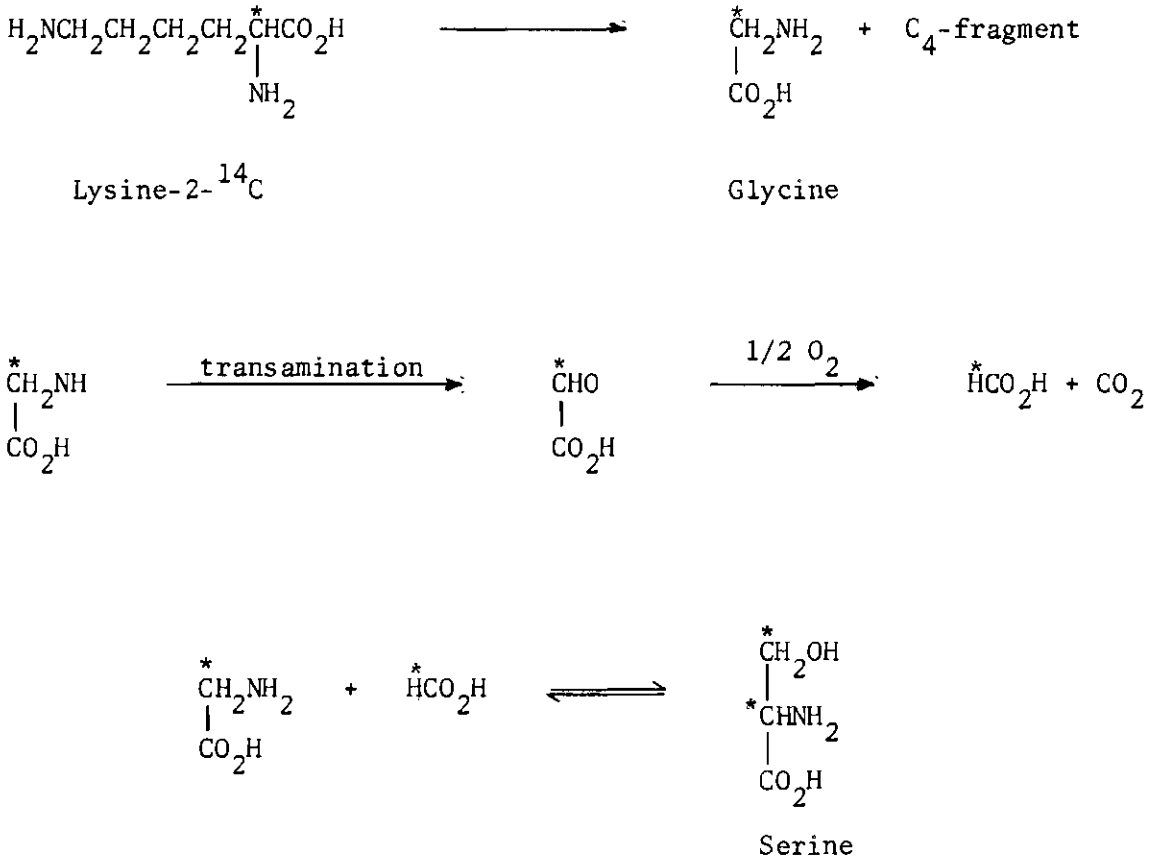
in a manner analogous to the synthesis of cysteine from serine by addition of hydrogen sulfide in a reaction catalyzed by a yeast enzyme (serine sulfhydrase) *(63,64)*.



The labelling patterns of the serine and diaminopropionic acid samples from viomycin with lysine-2-C^{14} as precursor are interesting in that a particular distribution of labelling has occurred to give approximately two-thirds of the activity at the C-2 position and one-third at the C-3 position. A conceivable pathway that would qualitatively explain such a labelling pattern is shown in Scheme 2. All transformations shown except the transformation from lysine to glycine are known *(65)*. The reversible interconversion of glycine and serine is well documented. It would be reasonable to expect that there would be more cold formate available than cold glycine so that the labelled formate would be diluted to a greater extent than the glycine. Thus the per cent activity at C-3 would be expected to be less than that at C-2.

In all four of the cases in which serine and diaminopropionic acid samples were degraded, the labelling patterns of the serine and diaminopropionic acid samples from the same sample of viomycin were virtually identical. These data, as shown in Table 11, offer convincing evidence that serine is the direct biological precursor of the diaminopropionic acid residue in viomycin.

Scheme 2



Since the experiment in which lysine-2-^{14}C was used as precursor indicated that lysine was the precursor of β-lysine in viomycin, it was decided that the determination of the per cent of the activity present at the C-2 position of the β-lysine would be informative. The only degradative reaction reported for β-lysine is the permanganate oxidation to give succinic acid *(9)*. The permanganate reaction would not yield exactly the information desired, and so it was decided to attempt a Barbier-Wieland degradation on a crystalline derivative of β-lysine, the methyl ester of di(N-phthalyl)-β-lysine as is outlined in Scheme 3. The methyl ester

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of the diphthalyl derivative of β-lysine resulting from lysine-2-^{14}C as the precursor was prepared by a modification of the method of van Tamelen *(9)*. The activity of this sample was determined to be 28,000 dpm per mg of the free base of β-lysine. The sample was diluted with 7.42 times as much of the methyl ester of the diphthalyl derivative prepared from nonradioactive β-lysine. The total sample was degraded by the usual Barbier-Wieland methods. The methyl ester was reacted with phenylmagnesium bromide, and the resulting tertiary alcohol was dehydrated in refluxing acetic anhydride. Ozonolysis of the resulting double bond, followed by oxidative workup, gave very low yields of di(N-phthalyl)ornithine and benzophenone.

The sample of di(N-phthalyl)ornithine was diluted with 4.76 times as much of nonradioactive di(N-phthalyl)ornithine prepared from ornithine by the method of van Tamelen *(9)*. Ornithine was then obtained by removal of the phthalyl protecting groups with hydrazine. A standard solution of this ornithine sample was prepared and an aliquot was reacted with ninhydrin in the same manner as was done in the degradation of serine and diaminopropionic acid samples.

The activities of the benzophenone, ornithine, and carbon dioxide from the ninhydrin reaction on ornithine were measured in the usual manner. These activities were corrected for the effects of the dilutions on the methyl ester of di(N-phthalyl)-β-lysine and on di(N-phthalyl)ornithine and then were converted to activities with respect to the equivalent amount of free base of β-lysine. These values could then be correlated to the positions in the β-lysine molecule from which they were derived. The activity of the C-1 position of β-lysine was calculated from the activity of the benzophenone to be 237 dpm/mg. The sum of the

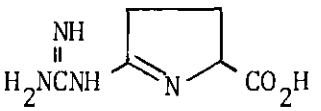
activities of the C-2 through the C-6 positions of β-lysine was calculated from the activity of the ornithine to be 29,100 dpm/mg. The activity of the C-2 position of β-lysine was calculated from the activity of the carbon dioxide obtained by the reaction of ninhydrin with ornithine to be 23,600 dpm/mg.

Based on the specific activity of the β-lysine itself of 28,000 dpm/mg, the partial labelling pattern of the β-lysine from viomycin having lysine-2-^{14}C as the precursor showed 0.8 per cent of the activity at C-1, 84.3 per cent at C-2, and the remainder of the activity at positions C-3 through C-6. The ornithine derived from β-lysine was calculated to contain 104 per cent of the activity of the β-lysine sample. This amount of error (5%) could easily have arisen as a result of the two dilutions of radioactive material that were necessary. This error does not affect the values for the labelling pattern to any significant extent, however.

These results demonstrate that lysine can serve as the precursor of β-lysine in viomycin.

Attempted Syntheses of 2-Guanidino-Δ^1-pyrroline-5-carboxylic Acid

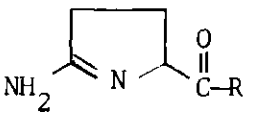
At the beginning of this research the structure 2-guanidino-Δ^1-pyrroline-5-carboxylic acid (VII) was the proposed structure for viomycidine, a guanidino amino acid that can be isolated from the acid hydrolysate of viomycin *(11, 17)*.



VII

It was felt that a synthesis of VII should be undertaken during the course of this research both to provide conclusive proof that this is the correct structure for viomycidine and to provide for a synthetic source of viomycidine to be used as cold carrier in any degradation of ^{14}C-labelled viomycidine to be undertaken.

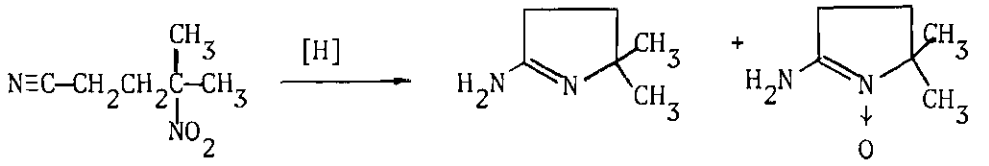
Since it was felt that 2-amino-Δ^1-pyrroline-5-carboxylic acid (XIV, R=OH) could be converted into VII by reaction with 1-guanyl-3,5-dimethylpyrazole *(11, 55, 67)*, all synthetic approaches basically involved developing a synthesis of XIV, an unknown compound.



XIV

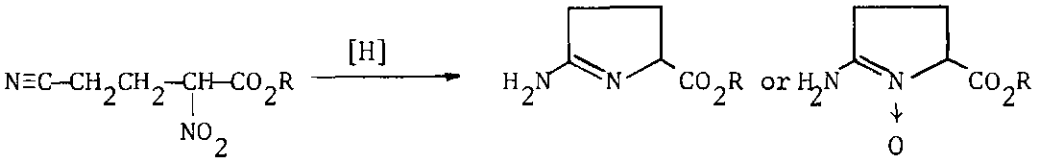
Although there are numerous approaches in the literature for the synthesis of Δ^1-pyrrolines, there are only a very limited number of approaches to the synthesis of 2-amino-Δ^1-pyrrolines. One synthetic approach reported that did yield 2-amino-Δ^1-pyrrolines involved the reductive cyclization of substituted 3-nitroalkyl cyanides *(68)*. It was reported, for example, that reduction of 4-nitro-4-methylvaleronitrile either catalytically (H_2, Raney nickel, 100°/100 atm) or chemically (iron and hydrochloric acid of zinc dust and ammonium chloride) gave 2-amino-5,5-dimethyl-Δ^1-pyrroline N-oxide (XV) and/or 2-amino-5,5-dimethyl-Δ^1-pyrroline (XVI) depending upon exact experimental

conditions. Moreover, the N-oxide (XVI) could be reduced further to give XV.



XVI XV

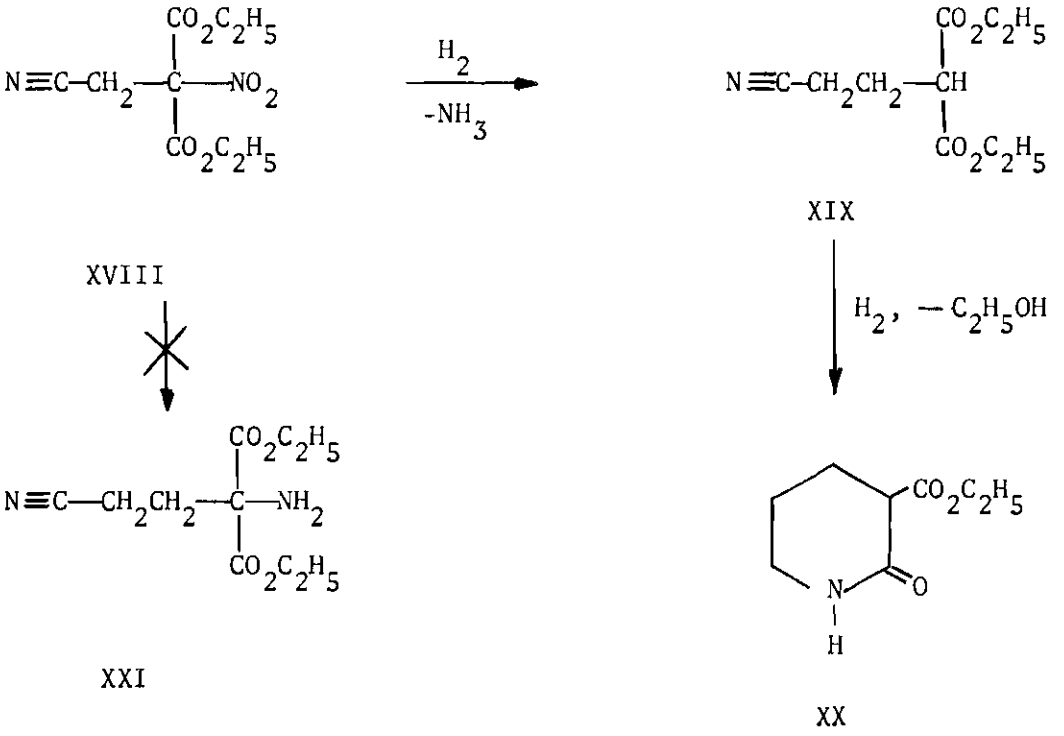
An analogous reductive cyclization using 4-cyano-2-nitrobutyric acid (XVII, R=H) would be expected to yield 2-amino-Δ^1-pyrroline-5-carboxylic acid and/or its N-oxide.



XVII XIV

Ethyl 4-cyano-2-nitrobutryrate (XVII, R=C_2H_5) was prepared from diethyl (2-cyanoethyl)nitromalonate (XVIII) as described elsewhere *(41)*. It was reported in the literature *(69)* that catalytic reduction of XVIII with either Raney nickel or palladium on charcoal in ethanol resulted in the hydrogenolysis of the nitro group before the cyano group was

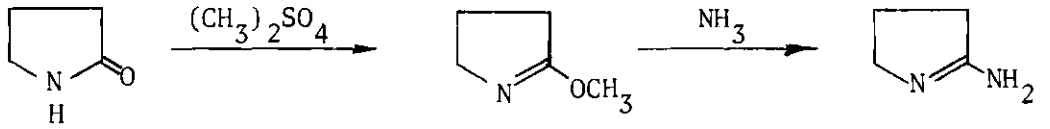
hydrogenated. The reduction could be controlled to give diethyl 2-cyanoethylmalonate (XIX) or 3-carbethoxy-2-piperidone (XX) but it was not found possible to stop the reduction at the desired diethyl amino(2-cyanoethyl)-malonate (XXI).



It was found experimentally that reduction of XVII ($R=C_2H_5$) with hydrogen and Raney nickel in methanol nickel in methanol at 20° and one atmosphere of pressure resulted in the uptake of 3.9 moles of hydrogen per mole of XVII reacted. Formation of the desired cyclization product, XIV, $R=OC_2H_5$, would have required an uptake of only three moles of hydrogen per mole of XVII reacted. It is probable that hydrogenolysis of the nitro group occurred since this reaction would require four moles of hydrogen per mole of XVII reacted, but the product was a dark oil with ill-defined n.m.r. and infrared spectra. No further identification was attempted.

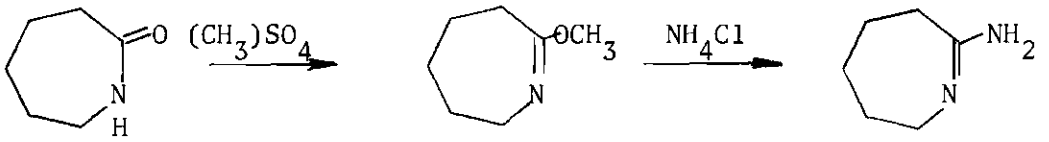
Several attempts were made to cyclize ethyl 4-cyano-2-nitrobutyrate reductively with zinc dust and acetic acid. Workup of the reduction mixture followed by chromatography over an IR-400 (OH^-) ion-exchange column yielded a small amount of an oil that darkened upon prolonged exposure to air. The n.m.r. and infrared spectra of this oil were ill-defined. Since the series of reactions described below had by this time yielded 2-amino-Δ^1-pyrroline-5-carboxamide (XIV, R=NH_2), the reduction of ethyl 4-cyano-2-nitrobutyrate was not pursued farther.

Peterson and Tietze reported that the reaction of 2-pyrrolidone with dimethyl sulfate can be made to yield 2-methoxy-Δ^1-pyrroline (XXII) *(42)*. Later Etienne and Correia reported *(70)* that the reaction of 2-methoxy-Δ^1-pyrroline with ammonia yielded 2-amino-Δ^1-pyrroline (XXXIII).



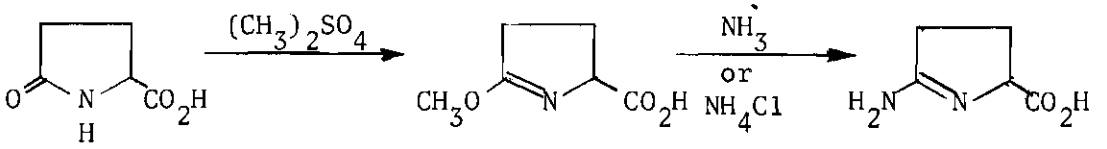
XXII XXIII

Benson and Cairns *(71)* described a similar preparation of XXIV from caprolactam, except they used ammonium chloride in the final step rather than ammonia.

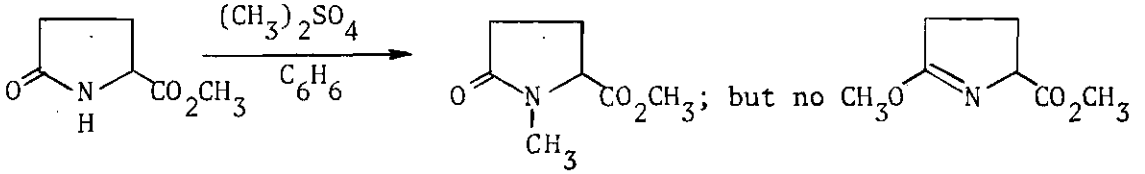


XXIV

An analogous set of reactions beginning with 2-pyrrolidone-5-carboxylic acid would yield the desired 2-amino-Δ^1-pyrroline-5-carboxylic acid. L-2-Pyrrolidone-5-carboxylic acid was prepared by a slight



modification of the method of Hardegger and Ott *(46)* by heating L-glutamic acid at 165° for several hours. It was found that preparation of the methyl ester gave greatly improved solubility characteristics over those of the 2-pyrrolidone-5-carboxylic acid. Numerous attempts were made to prepare methyl 2-methoxy-Δ^1-pyrroline-5-carboxylate (XXV) by reactions of methyl-2-pyrrolidone-5-carboxylate with dimethyl sulfate using both the methods of Peterson and Tietze *(42)* and Benson and Cairnes *(71)*, but only methyl N-methyl-2-pyrrolidone-5-carboxylate (XXVI) could



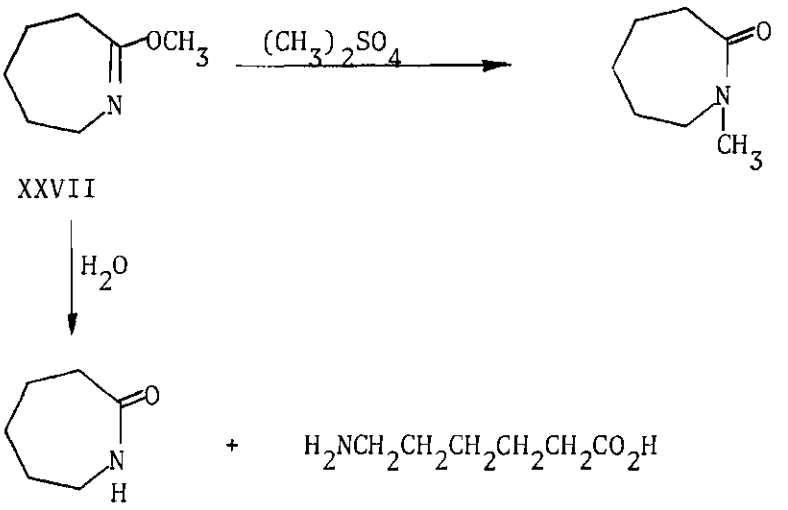
XXVI XXV

be detected or isolated. The reaction of methyl iodide and silver oxide with methyl-2-pyrrolidone-5-carboxylate was also investigated. Although only methyl N-methyl-2-pyrrolidone-5-carboxylate could be isolated from the reaction mixture, an analysis of the n.m.r. spectrum of the reaction mixture before workup indicated that a maximum of 18 per cent of methyl 2-methoxy-Δ^1-pyrroline-5-carboxylate was present in the reaction mixture.

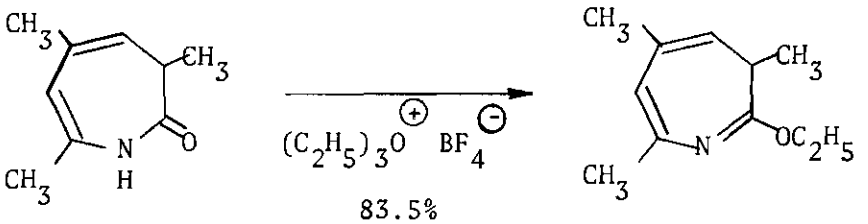
In order to investigate further the conditions under which O-methylation rather than N-methylation could be achieved, 2-pyrrolidone was reacted with dimethyl sulfate by the method of Peterson and Tietze *(42)*. A 13 per cent yield of 2-methoxy-Δ^1-pyrroline was obtained without any difficulty. Some N-methyl-2-pyrrolidone was also obtained, but the great loss in yield was largely due to the large amount of tar that formed in the distillation pot.

Benson and Cairnes reported *(71)* that N-methylcaprolactam could be obtained from caprolactam by reaction with a 0.3 molar excess of dimethyl sulfate or from O-methylcaprolactam (XXVII) by heating with a small amount of dimethyl sulfate or by heating (XXVII) in a steel bomb at 285° for 10 days. Benson and Cairnes also reported that by simply heating a solution of XXVII in water under reflux, it was converted into caprolactam (15%) and ε-aminocaproic acid (45%).

It is possible that any methyl 2-methoxy-Δ^1-pyrroline-5-carboxylic acid formed in the reaction with dimethyl sulfate either was hydrolyzed during aqueous workup or was isomerized to the N-methyl-2-pyrrolidone-5-carboxylic acid during distillation.

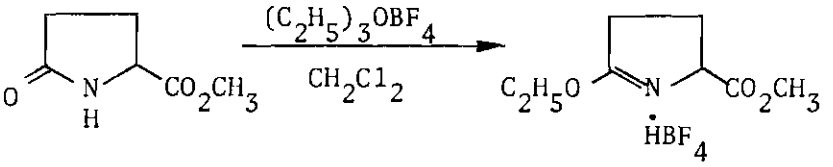


Paquette *(72)* reported that dimethyl sulfate was ineffective for the conversion of the amide function of 1,3-dihydro-3,5,7-trimethyl-2H-azipinone-2 (XXVIII) into the O-methyl imino ether derivative, but he reported that the use of triethyloxonium fluoborate readily yielded the O-ethyl imino ether derivative XXIX. The reagent was prepared by



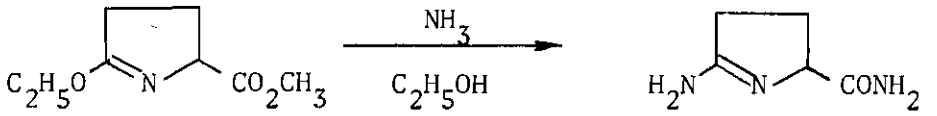
XXVIII XXIX

the method of Meerwein *(48)* and reacted with methyl 2-pyrrolidone-5-carboxylate in methylene chloride for two hours at room temperature. After evaporation of the solvent, a clear oil remained. Analysis of the oil by n.m.r. indicated that the desired reaction had occurred and that methyl 2-ethoxy-Δ^1-pyrroline-5-carboxylate had been formed in practically quantitative yield.



The methyl 2-ethoxy-Δ^1-pyrroline-5-carboxylate was divided into two portions and was reacted without further purification with both a saturated solution of ammonia in ethanol and guanidine hydrochloride in ethanol. The reaction mixtures were allowed to stand. A white solid was obtained from the reaction with guanidine hydrochloride. This solid gave a negative Sakaguchi test and therefore contained none of the desired ethyl 2-guanidino-Δ^1-pyrroline-5-carboxylate. Work-up of the reaction with ammonia yielded a colorless oil that partially solidified upon trituration with methylene chloride. After conversion of this compound from the fluoborate salt to the chloride salt, it was re-crystallized from ethanol-water. The n.m.r. spectrum indicated that the

compound formed was 2-amino-Δ^1-pyrroline-5-carboxamide hydrochloride. Elemental analysis confirmed that ammonolysis of the ester



had occurred.

The compound was optically inactive. Since the starting material, L-2-pyrrolidone-5-carboxylic acid, was optically active, racemization occurred during one of the subsequent reactions. The proton on the asymmetric carbon should be relatively acidic and reaction with ammonia in ethanol at room temperature for three days apparently caused racemization.

Since a derivative of the desire compound, 2-amino-Δ^1-pyrroline-5-carboxylic acid, had now been synthesized, it was necessary to consider conversion of the amino group to a guanidino group. Bannard and coworkers investigated the three most common methods for converting amino groups to guanidino groups *(73)*: reaction of amine salts with cyanamide in aqueous or alcoholic solutions, reaction of amines with 5-methyliso-

thiouronium sulfate, and reaction of amines with 1-guanyl-3,5-dimethylpyrazole nitrate. Bannard and co-workers reported that the third method was superior to the other two in at least three important aspects: the reaction proceeds under milder conditions, much better yields were obtained, and a wider variety of amino compounds could be reacted.

Fasold and co-workers *(66)* reported experimental conditions for conversion of amino acids to the corresponding guanidino acids by reaction with 1-guanyl-3,5-dimethylpyrazole. 2-Amino-Δ^1-pyrroline-5-carboxamide proved to be inert to 1-guanyl-3,5-dimethylpyrazole under these conditions, however. Scott and co-workers *(67)* reported that reaction of amines with 1-guanyl-3,5-dimethylpyrazole nitrate proceeded in boiling water. 2-Amino-Δ^1-pyrroline-5-carboxamide proved to be inert under these conditions.

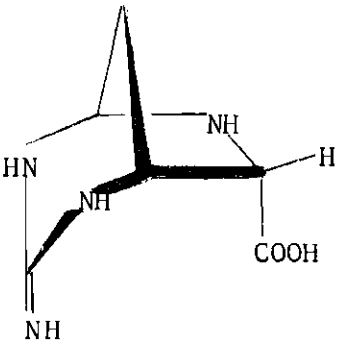
If viomycidine has structure VII, then 2-amino-Δ^1-pyrroline-5-carboxamide should have an n.m.r. spectrum similar to that of viomycidine, at least with respect to the protons bound to carbon. The n.m.r. spectrum in D_2O was reported by Miller *(11, 16)* to consist of peaks at τ7.43 (2H, triplet, $\underline{J}$ = 2.1 cps), 5.38 (2H, closely spaced multiplet), and 4.37 (1H, triplet, $\underline{J}$ = 2.2 cps). The n.m.r. spectrum of 2 amino-Δ^1-pyrroline-5-carboxamide in D_2O was radically different: absorptions at 6.80-8.11 (complex multiplet, 4H) and 5.32 (6H, HOD peak superimposed over a peak with a weight of one proton) were present.

Because of the lack of similarity between these two spectra, some question was raised as to whether VII was the correct structure for viomycidine. Since VII could not be obtained from 2-amino-Δ^1-pyrroline-5-carboxamide, the synthetic approach did not appear to offer a solution to the question of a correct structure for viomycidine. At this time

it was decided that X-ray crystallography offered the most promising approach to the determination of the correct crystal structure of viomycidine.

The Crystal Structure of Viomycidine Hydrobromide

The structure determined for viomycidine by X-ray diffraction techniques was 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid (XXX).



XXX

A view of the viomycidine cation projected onto the XY plane is shown in Figure 11. Table 12 lists the calculated *(74)* bond distances and bond angles in the structure. Estimated standard deviations in the last significant digit are given in parentheses for each of the calculated values.

The bond distances in the five-membered ring are slightly longer than normal values for C-N and C-C bonds, but these distances are consistent with the slightly longer bond distances typically found in the five-membered rings in tricyclo[3,2,1,$0^{2,4}$]octane *(75)* and tricyclo [5.2.1.$0^{2,6}$]deca-3,8-diene *(76)* nuclei. All other bond distances have normal values, and even the slightly longer bond distances

Table 12. Bond Distances and Bond Angles in the Viomycidine Cation

Bond Distances		Bond Angles	
Atoms	Bond Distance (Å)	Atoms	Bond Angle (deg.)
O(1)-C(1)	1.25 (4)	N(4)-C(6)-N(2)	120 (2)
O(2)-C(1)	1.23 (4)	N(4)-C(6)-N(3)	120 (4)
N(1)-C(2)	1.58 (4)	N(3)-C(6)-N(2)	119 (3)
N(1)-C(5)	1.56 (5)	C(6)-N(2)-C(5)	124 (3)
N(2)-C(5)	1.46 (4)	N(2)-C(5)-C(4)	106 (3)
N(2)-C(6)	1.44 (4)	N(2)-C(5)-N(1)	105 (3)
N(3)-C(3)	1.45 (4)	C(5)-C(4)-C(3)	99 (2)
N(3)-C(6)	1.39 (4)	N(1)-C(2)-C(3)	102 (3)
N(4)-C(6)	1.31 (4)	C(5)-N(1)-C(2)	109 (3)
C(1)-C(2)	1.43 (4)	N(1)-C(2)-C(1)	109 (3)
C(2)-C(3)	1.66 (5)	C(2)-C(1)-O(1)	118 (4)
C(3)-C(4)	1.60 (4)	C(2)-C(1)-O(2)	116 (3)
C(4)-C(5)	1.60 (4)	O(1)-C(1)-O(2)	126 (4)
		C(2)-C(3)-C(4)	102 (2)
		C(2)-C(3)-N(3)	112 (3)
		C(4)-C(3)-N(3)	110 (3)
		C(3)-N(3)-C(6)	117 (3)
			Dihedral Angle (deg).
		N(4)-C(6)-N(2) N(4)-C(6)-N(3)	175 (6)
		C(2)-C(1)-O(1) C(2)-C(1)-O(2)	170 (6)

in the five-membered ring are less than two standard deviations from normal values.

The bridgehead angle C(3)-C(4)-C(5) value of 99° can be compared with the value of 97° found for the corresponding angle in the tricyclo [3,2,1,$0^{2,4}$]octane nucleus *(75)*. All other bond angles show normal values. The dihedral angles between the four atoms in each of the two trigonal groups are less than one standard deviation from the normal value of 180°.

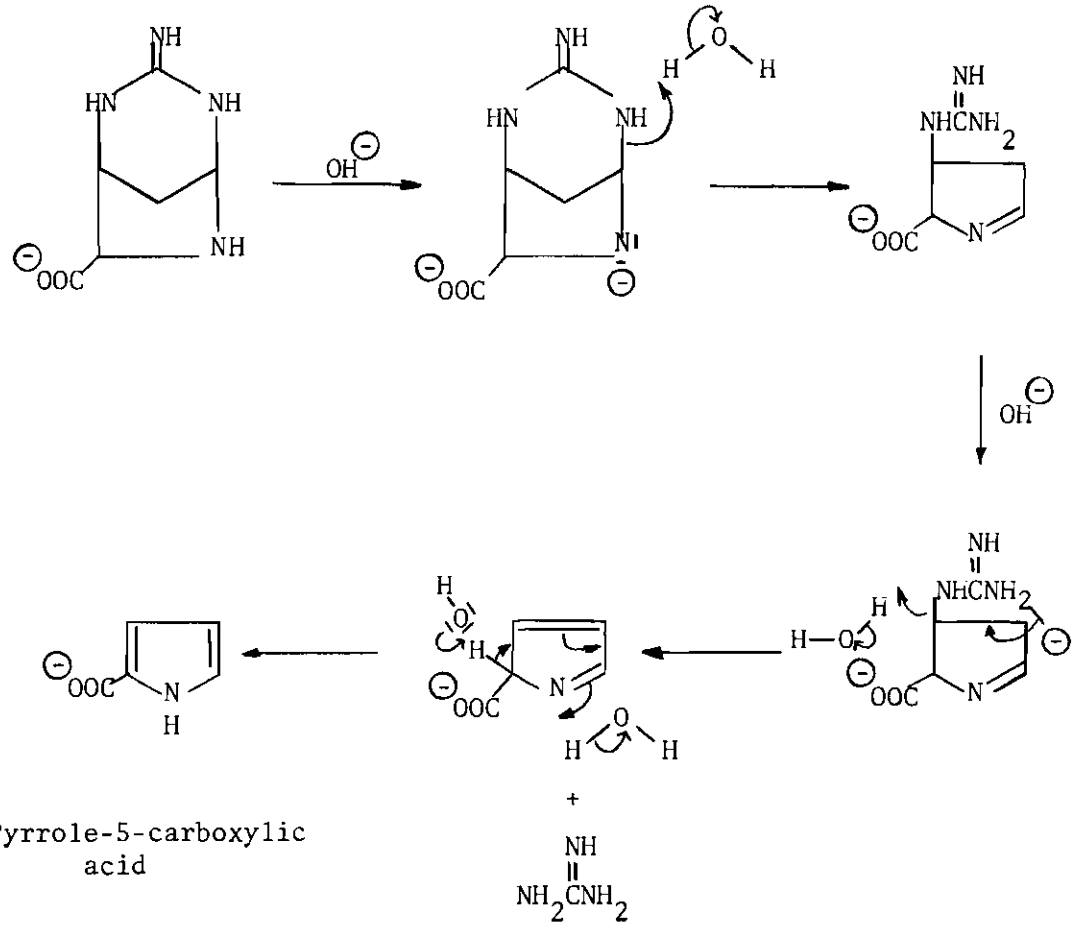
The bromide anion is located in the crystal structure almost halfway between the guanidino function of one cation and the N(1) amino function of an adjacent cation; the closest interatomic distances was 3.55 Å for Br-C(6) and 3.37Å for Br-N(1). The bromide anion is located on the opposite side of the guanidino function from the carboxylate function.

Shortly after crystal structure of viomycidine had been determined, Johnson and coworkers *(77)* suggested the same structure for viomycidine based on a private communication from Professor G. Büchi.

In sharp contrast to the stability of viomycidine to acid (it is obtained from treatment of viomycin in 6 N hydrochloric acid on the steam bath for twenty hours) is its apparent lability in base. It had been reported that hydrolysis of viomycidine by hot barium hydroxide solution for 77 hours furnished nearly three moles of ammonia and a 21 per cent yield of pyrrole-2-carboxylic acid *(13, 16)*. It had also been reported that sodium hydroxide fusion of viomycidine gave 2-amino-pyrimidine, pyrrole-2-carboxylic acid, and glycine *(13)*. The formation of pyrrole-2-carboxylic acid from XXX requires merely the elimination of

a guanidine unit and rearrangement of double bonds. Further hydrolysis of the guanidine unit would yield carbon dioxide and three moles of ammonia. On the other hand, base catalyzed elimination of glycine from XXX would yield 2-aminopyrimidine. These transformations may be postulated as occurring as shown below in Scheme 4.

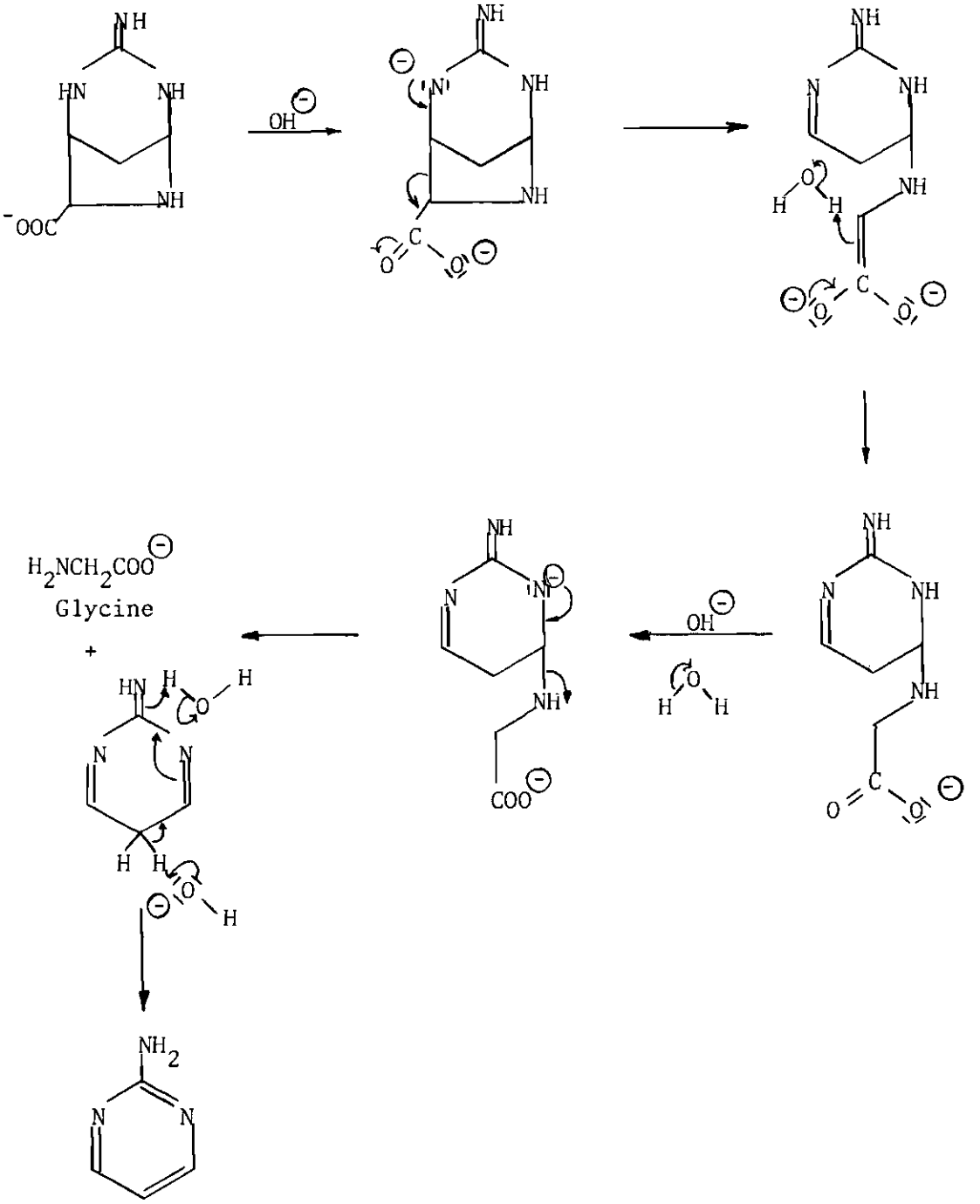
Scheme 4



Pyrrole-5-carboxylic acid

Guanidine

Scheme 4 (continued)

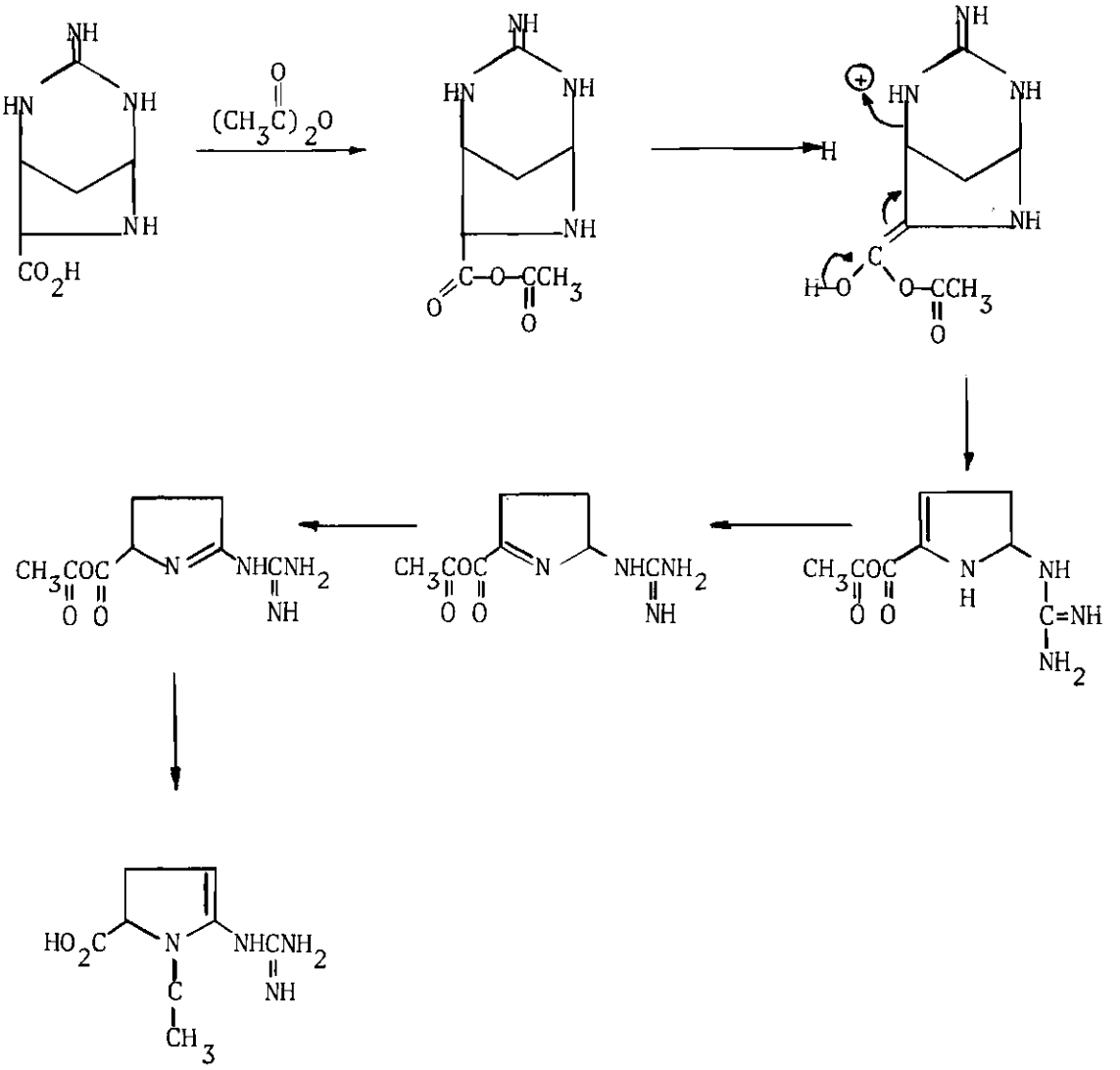


2-Aminopyrimidine

Nasser *(11)* reported that acetylation of viomycidine in hot pyridine and actic anhydride, followed by ozonolysis, oxidation of the ozonide, and hydrolysis yielded DL-aspartic acid in 52 per cent yield. The fact that DL-rather than D- or L-aspartic acid was obtained was attributed to racemization by pyridine during acetylation. Miller *(16)* had previously reported the isolation and characterization of an optically active acetyl derivative of viomycidine by reaction of viomycidine with acetic anhydride in ethanol-water solvent at 10°. It was tacitly assumed that the only difference between Miller's acetylviomycidine and Nasser's acetyl derivative of viomycidine was in the optical activity (Nasser did not isolate acetylviomycidine but instead he ozonolyzed directly the crude reaction mixture). It was therefore suggested early in this research that the absolute configuration of viomycidine would be determined by carrying out Nasser's degradation sequence on optically active acetylviomycidine. Optically acetive acetylviomycidine was prepared as described by Miller *(16)* and degraded as described by Nassar *(11)*. There was obtained by preparative paper chromatography a very low yield of a solid which was ninhydrin positive and which had the same R_F value as aspartic acid in BAW. This solid could not be crystallized, and the infrared spectrum was different from the infrared spectra of either L-aspartic acid or DL-aspartic acid. The attempt to obtain aspartic acid from the acetylviomycidine prepared by Miller's method was repeated several times with similar results: at most only a trace of aspartic acid was obtained. These apparently contradictory results can be reconciled by noting that it is probable that the product Nassar obtained by acetylation in hot pyridine was not the same compound that

Miller had obtained by acetylation in ethanol-water. Since viomycidine appears to be labile in the presence of bases, it is possible that the acetylation of viomycidine in the presence of hot pyridine actually does yield the Nacetylated structure (XXI) proposed by Nassar as shown in Scheme 5. Ozonolysis, oxidation, and hydrolysis of XXXI would then be

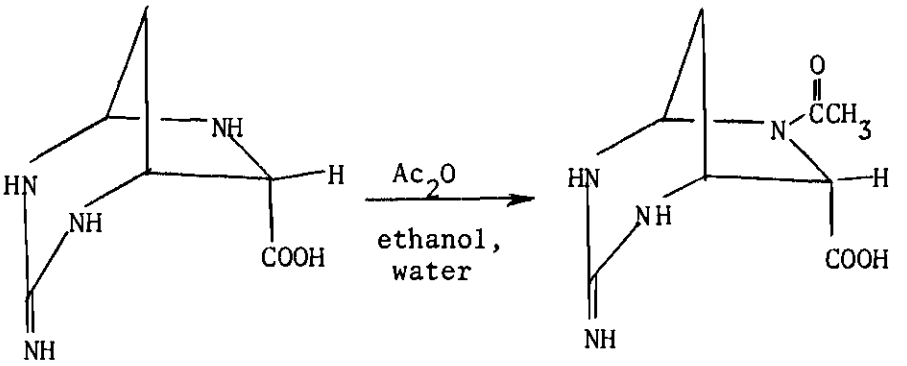
Scheme 5



XXXI

expected to yield DL-aspartic acid as was described by Nassar.

Acetylation of viomycidine with acetic anhydride in ethanol-water would be expected to yield the N-acetylated product (XXXII) without the ring opening that probably occurs in the presence of base. The n.m.r.



XXXII

spectrum of N-acetylviomycidine prepared from acetic anhydride in ethanol-water is in agreement with structure XXXII. This n.m.r. spectrum will be discussed in detail later in conjunction with a discussion of the n.m.r. spectrum of viomycidine.

The fact that viomycidine gave a positive Sakaguchi test led earlier workers to assign structures to viomycidine having monosubstituted guanidino groups. Since the Sakaguchi test is carried out in alkaline media, it appears that during the Sakaguchi test viomycidine reacts to give a monosubstituted guanidino compound, which then can give a positive Sakaguchi test.

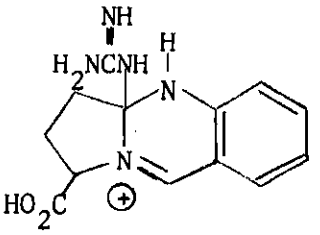
The pink color that viomycidine gives with Weber reagent is

normally indicative of a monosubstituted or N,N-disubstituted guanidine compound *(10,32)*. N,N'-Disubstituted guanidines give colors ranging from blue to purple. Since the Weber test is carried out in basic media, it may be that viomycidine reacts to give a monosubstituted guanidine before giving a positive Weber test.

The pKa values *(10)* of 2.8, 5.87, and 13.4 in 66 per cent dimethylformamide and of 5.50 and 12.6 in water are consistent with structure XXX for viomycidine. as these values require a strongly acidic group, a strongly deactivated amino group, and a guanidino group. The carboxyl group would be expected to be strongly acidic due to the presence of an α-amino group and a β-guanidino group. The amino group would be expected to be deactivated by the nitrogen of the guanidino group that is attached to the same carbon as the amino nitrogen.

The fact that the amino group of viomycidine exhibits a differential ultraviolet absorption spectrum, which was taken to indicate that it was a tertiary amino group *(10)*, might have resulted from the instability of viomycidine in the basic solution, pH 9.80, used in one of the cells.

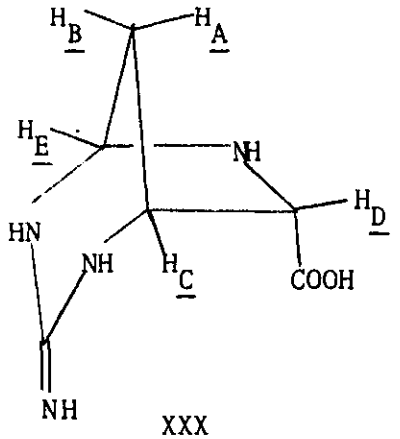
One test for which it is difficult to explain the results based on structure XXX is the positive test given by viomycidine to o-aminobenzaldehyde reagent *(16)*. This reagent, which is weakly acidic, gives a stable yellow color with Δ^1-pyrrolines and imines. The formation of a structure of the type XXXIII, which is probably responsible for the yellow color, might be the driving force for opening the viomycidine structure to give a Δ^1-pyrroline structure.



XXXIII

The absorptions observed in the n.m.r. spectrum of viomycidine (XXX) in deuterine oxide solution by Miller *(11,16)* are assigned as shown below. It was observed by Miller *(78)* that when the n.m.r. spectrum of viomycidine was determined in deuterium oxide containing sodium bicarbonate, the low field proton disappears and absorptions appear at τ 5.5-5.9, 6.0-6.1 and 7.7-7.9, all complex multiplets. This fact, together with the other observed properties of viomycidine in weakly or strongly alkaline solution, suggests that viomycidine in these alkaline solutions behaves as if it had the structure 2-guanidino-Δ^1-pyrroline-5-carboxylic acid.

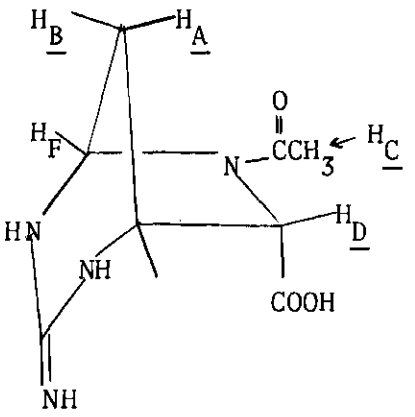
The peaks observed in the n.m.r. spectrum of crystalline acetyl-



XXX

H	τ	J_1 cps
A,B	7.43	2.1 (triplet) closely spaced
C,D	5.38	multiplet
E	4.37	2.2 (triplet)

viomycidine (XXXII) in deuterium oxide solution as observed by Miller *(16)* are assigned as shown below.



XXXII

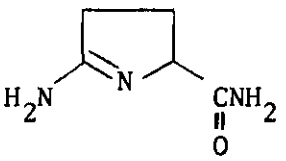
H	τ
A,B	7.73, closely spaced multiplet
C	7.73, singlet
D,E	5.29-5.68, multiplet
F	4.19-4.44, multiplet

CHAPTER IV

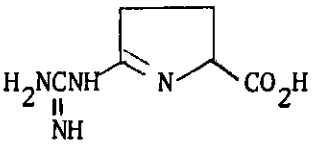
CONCLUSIONS

Radioactively labelled glucose, serine, lysine, and arginine have been shown to be incorporated into the viomycin molecule relatively efficiently when added to cultures of Streptomyces griseus var. purpurea on the third day after inoculation. Serine has been shown to be the direct biosynthetic precursor of the serine and α,β-diaminopropionic acid residues in viomycin. The results linking serine and diaminopropionic acid have been published in communication form *(79)*. Lysine has been shown to be the precursor of the β-lysine residue in viomycin, and a pathway from lysine to serine and diaminopropionic acid has been demonstrated. Arginine has been shown to be a precursor of the viomycidine residue in viomycin. Glutamic acid and aspartic acid have been found not to be incorporated into the viomycin molecule to any significant extent.

2-Amino-Δ^1-pyrroline-5-carboxamide (XIV) has been synthesized and characterized. The nuclear magnetic resonance spectrum of XIV is grossly different from the spectrum of viomycidine, thus casting doubt on the correctness of the previously suggested structure (VII) for viomycidine.

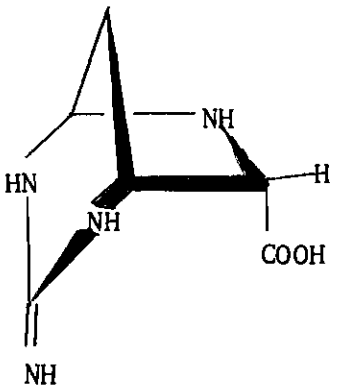


XIV



VII

The crystal structure of viomycidine hydrobromide has been determined by X-ray diffraction techniques to be 2,4,6-triaza-3-imino-bicyclo[3.2.1]octane-7-carboxylic acid (XXX). The results of this structure determination have been published in communication form *(80)*.



XXX

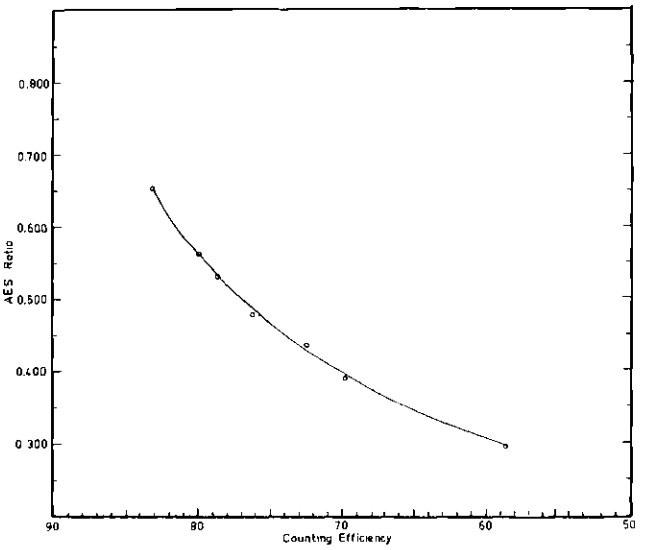


Figure 1. Quenching Curve for the Water-Hydrochloric Acid-Toluene-Triton X-100 Scintillation System

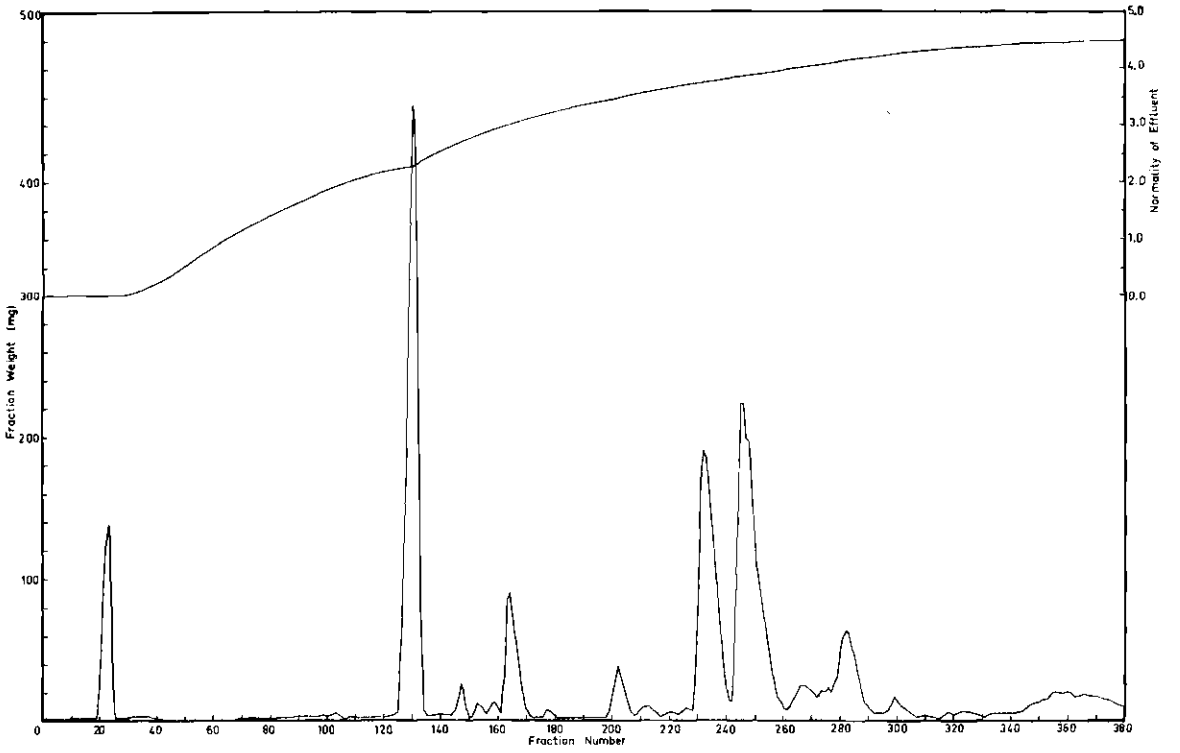


Figure 2. Weight Curve for the Gradient Elution Chromatography of the Hydrolysate of Viomycin.

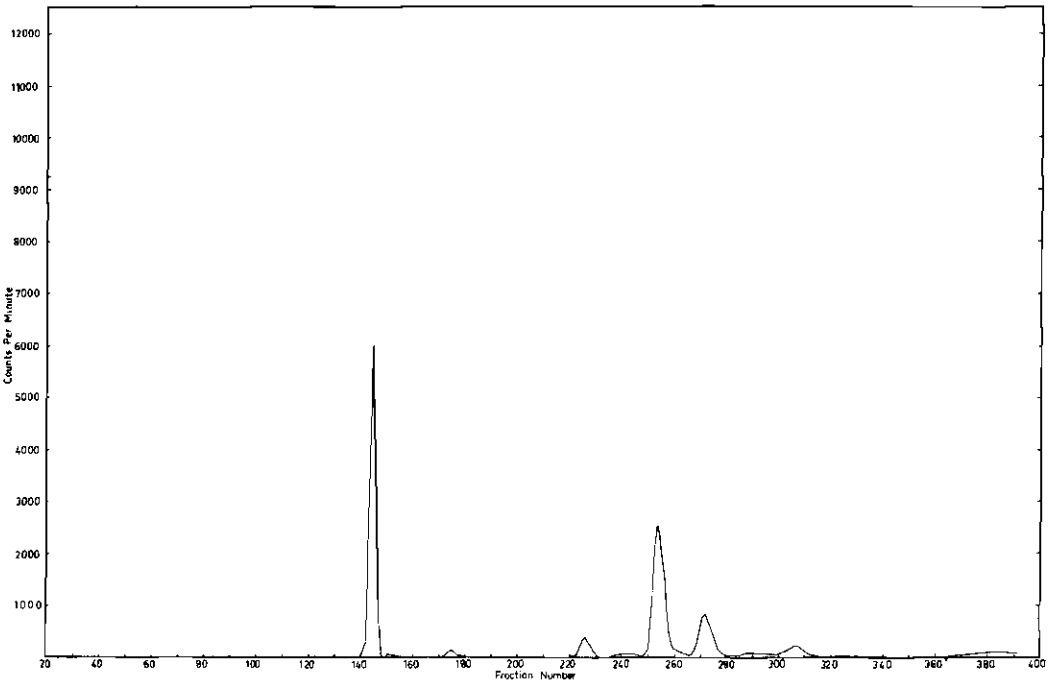


Figure 3. Activity Curve for the Hydrolysate of Viomycin from D-Glucose-U-^{14}C (Run No. I)

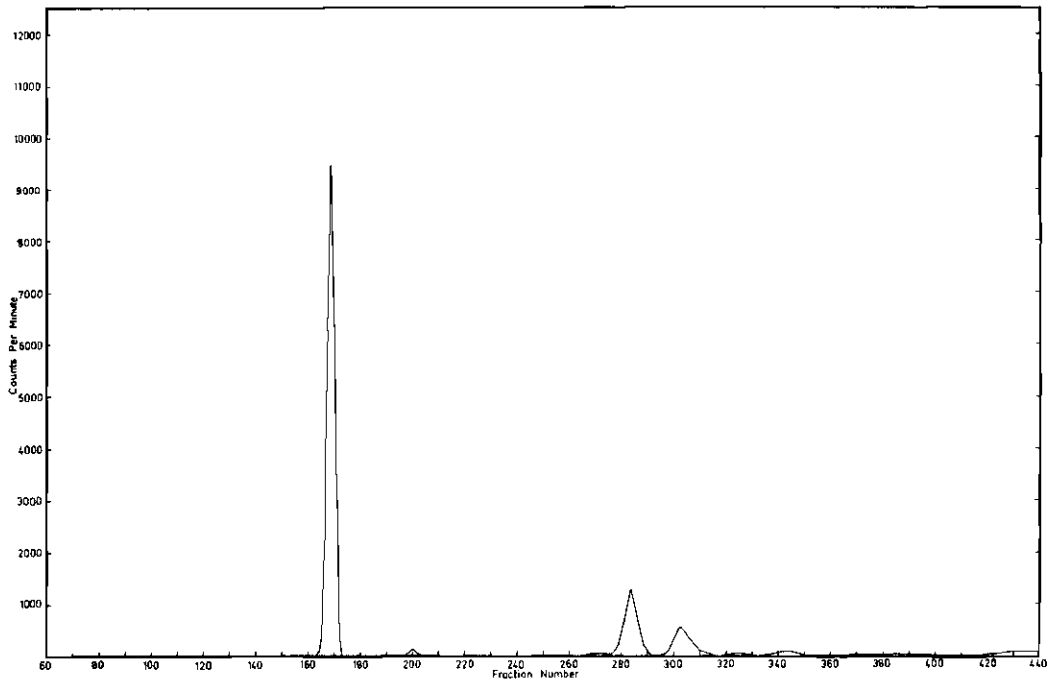


Figure 4. Activity Curve for the Hydrolysate of Viomycin from DL-Serine-3-^{14}C (Run No. II)

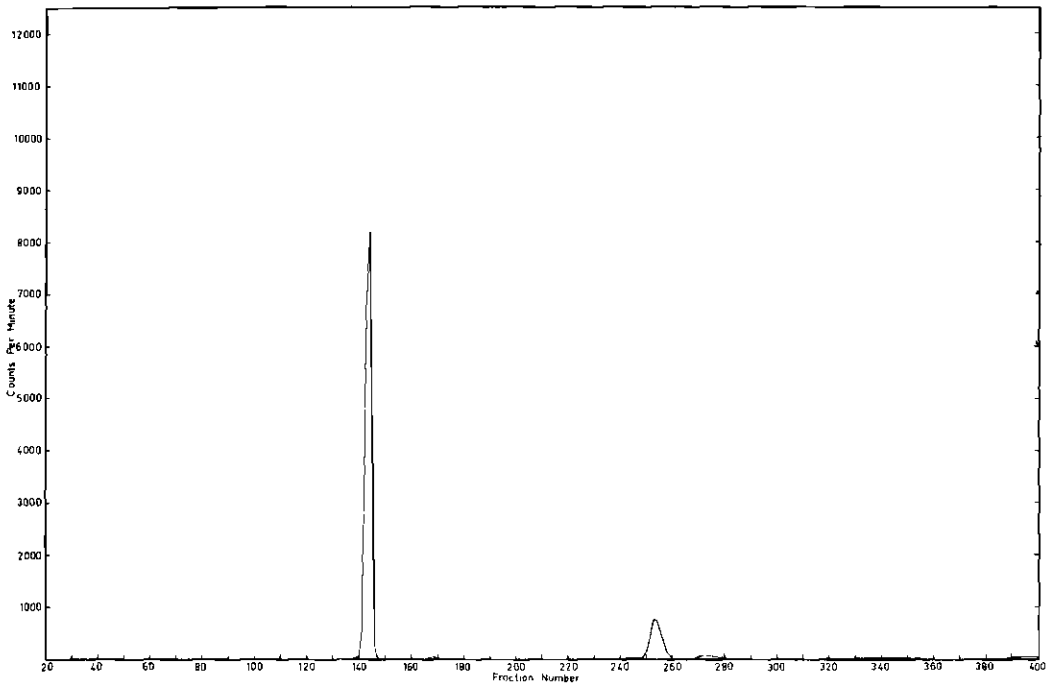


Figure 5. Activity Curve for the Hydrolysate of Viomycin from DL-Serine-1-^{14}C (Run No. III)

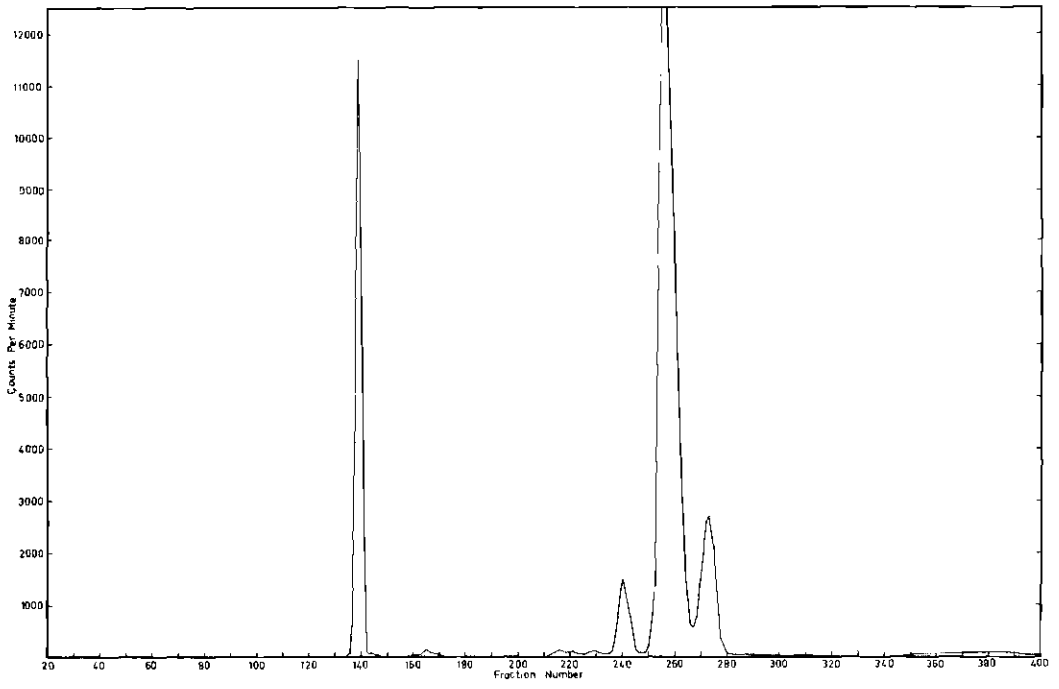


Figure 6. Activity Curve for the Hydrolysate of Viomycin from DL-Lysine-2-^{14}C (Run No. IV)

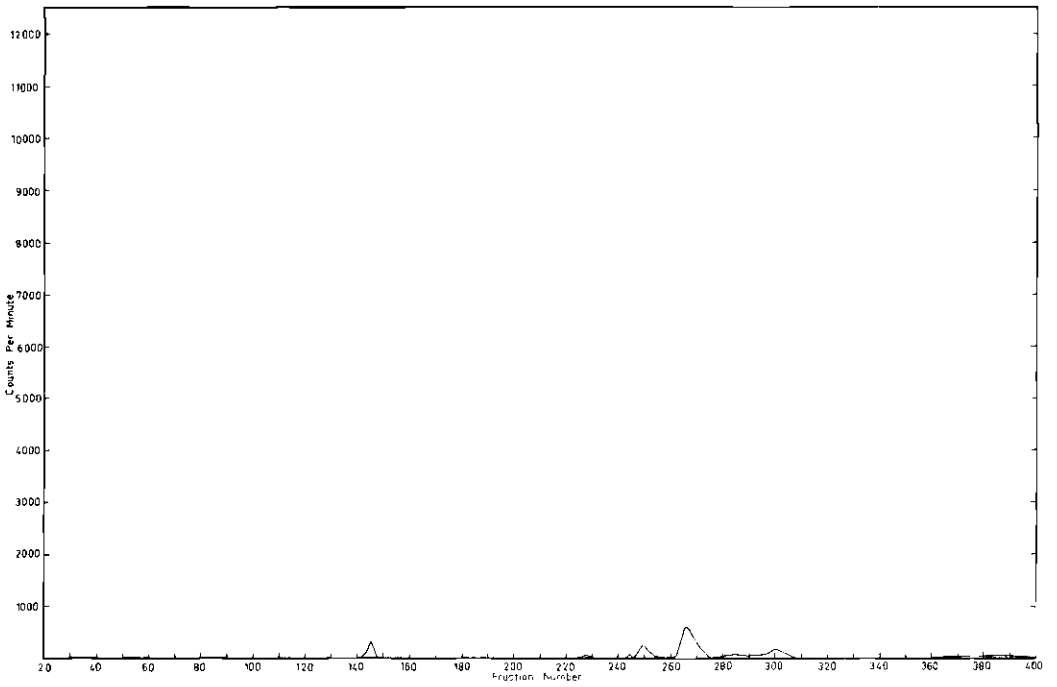


Figure 7. Activity Curve for the Hydrolysate of Viomycin from DL-Glutamic-3,4-^{14}C Acid (Run No. V)

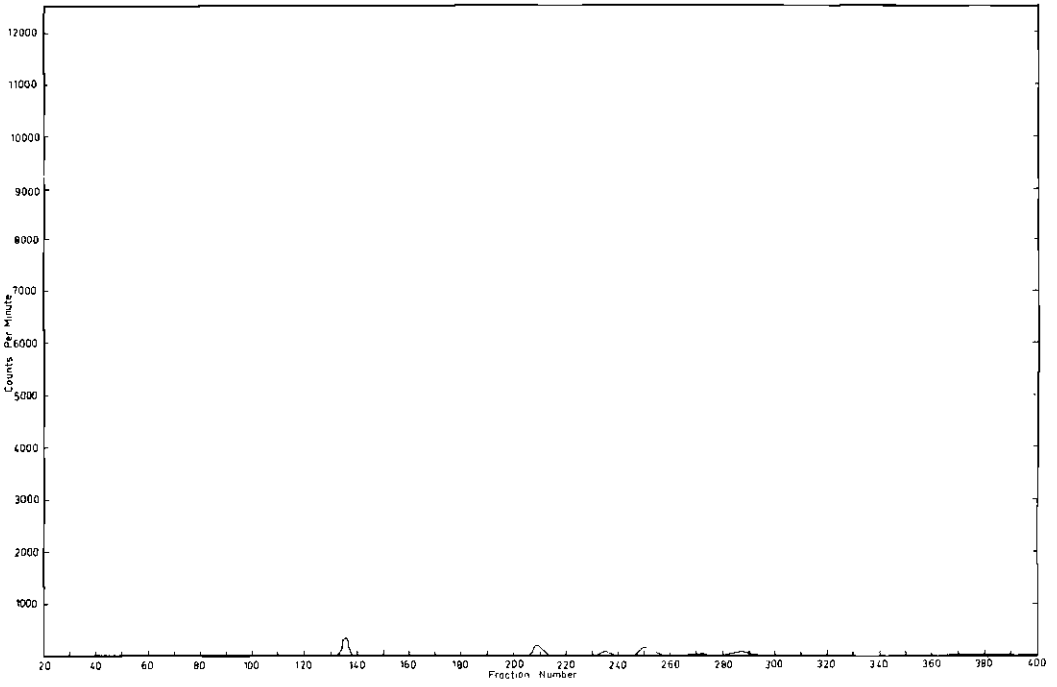


Figure 8. Activity Curve for the Hydrolysate of Viomycin from DL-Glutamic-5-^{14}C Acid (Run No. VI)

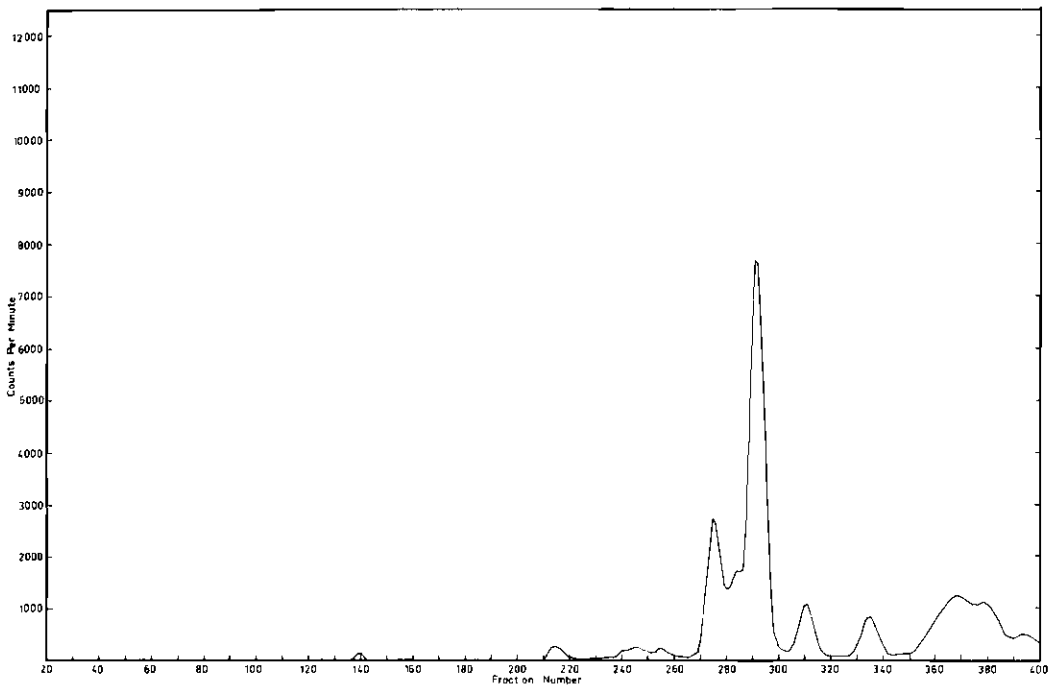


Figure 9. Activity Curve for the Hydrolysate of Viomycin from DL-Arginine-5-^{14}C (Run No. VII)

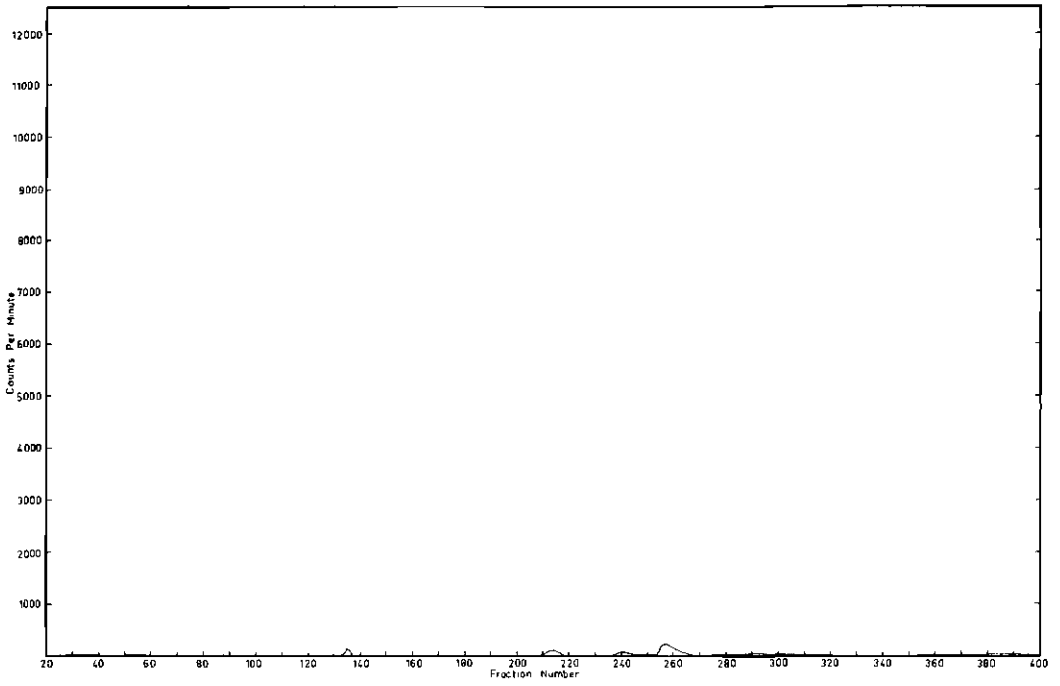


Figure 10. Activity Curve for the Hydrolysate of Viomycin from DL-Aspartic-4-^{14}C Acid (Run No. VIII)

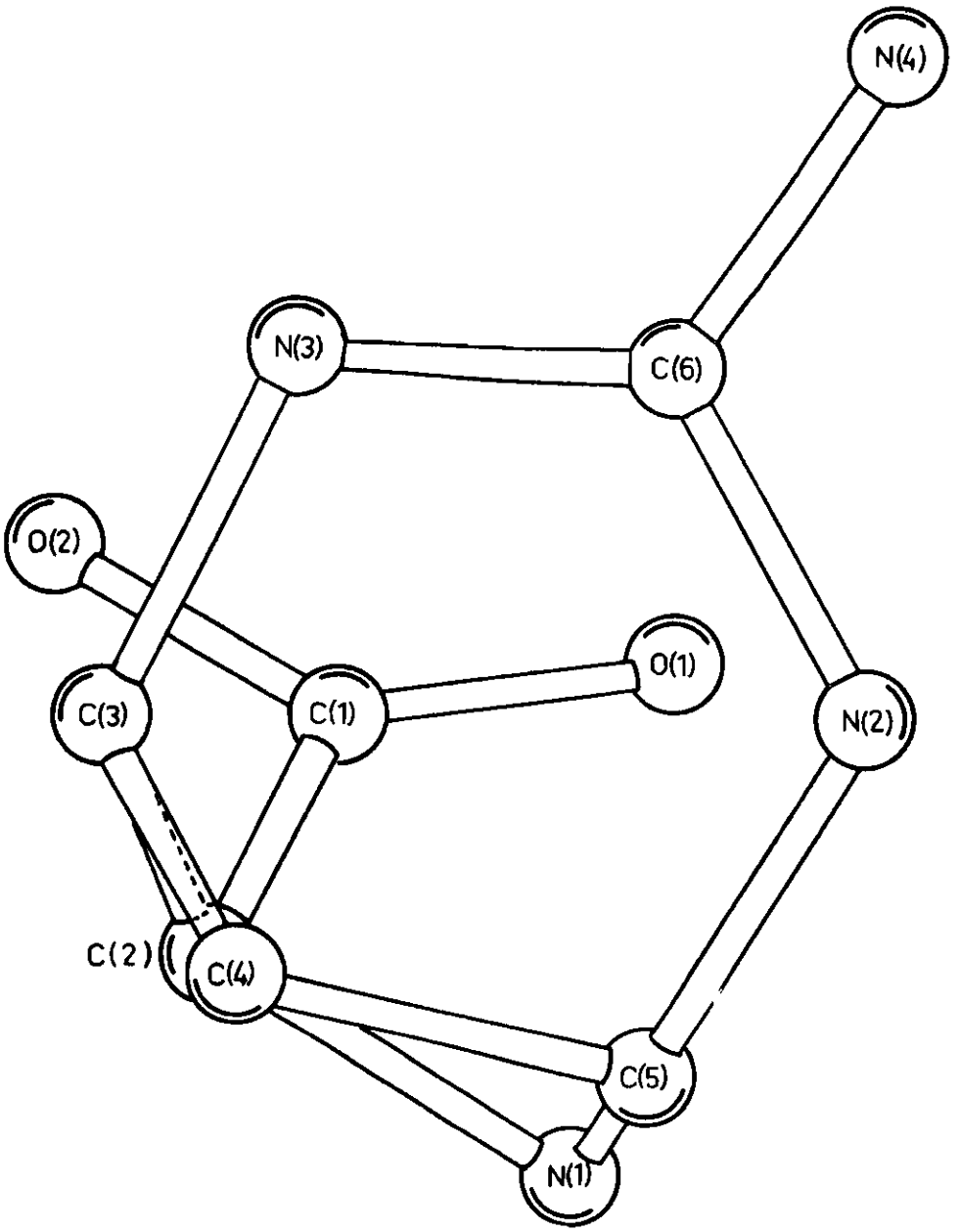


Figure 11. Structure of the Viomycidine Cation in Crystalline Viomycidine Hydrobromide

LITERATURE CITED*

1. A. C. Finlay, G. L. Hobby, F. Hochstein, T. M. Lees, T. F. Lenert, J. A. Means, S. Y. P'An, 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. T. H. Haskell, S. A. Fusari, R. P. Frohardt, and Q. R. Bartz, *J. Am. Chem. Soc., 74,* 599 (1952).

5. L. H. Mason, Ph.D. Thesis, University of Illinois, 1953.

6. C. K. Kellogg, Ph.D. Thesis, Georgia Institute of Technology, 1963.

7. M. D. Cohen and E. Fischer, *J. Chem. Soc., 1962,* 3044.

8. T. Kitagawa, Y. Sawada, T. Miura, T. Ozasa and H. Taniyama, *Tetrahedron Letters,* 109 (1968).

9. E. E. van Tamelen and E. E. Smissman, *J. Am. Chem. Soc., 75,* 2031 (1953).

10. H. B. Hayes, M.S. Thesis, Georgia Institute of Technology, 1959.

11. R. F. Nassar, Ph.D. Thesis, Georgia Institute of Technology, 1964.

12. J. H. Bowie, D. A. Cox, A. W. Johnson and G. Thomas, *Tetrahedron Letters,* 3305 (1964).

13. J. R. Dyer, H. B. Hayes, and E. G. Miller, Jr., Paper presented at the Third Interscience Conference for Antimicrobial Agents and Chemotherapy, Washington, D. C. (1963).

14. J. H. Bowie, A. W. Johnson, and G. Thomas, *Tetrahedron Letters,* 863 (1964).

15. J. R. Dyer, C. K. Kellogg, R. F. Nassar, and W. E. Streetman, *Tetrahedron Letters,* 585 (1965).

---

*For the complete titles of all journals referred to see *Chemical Abstracts, 50,* 1 J(1956).

16. E. G. Miller, Jr., Ph.D. Thesis, Georgia Institute of Technology, 1963.

17. J. R. Dyer, H. B. Hayes, E. G. Miller, Jr., and R. F. Nassar, *J. Am. Chem. Soc., 86,* 5363 (1964).

18. Alton Meister, "Biochemistry of the Amino Acids", Vol II, Academic Press, Inc., New York, 1965, pp. 636-673.

19. R. Gamelin, G. Strauss, and G. Hasenmaier, *Z. Physiol. Chem., 314,* 28 (1959).

20. P. R. Adiga, S. L. N. Rao, and P. S. Sarma, *Current Sci. (India), 32,* 153 (1963).

21. E. A Bell and A. S. L. Tirimanna, *Biochem. J., 97,* 104 (1965).

22. G. Roncari, Z. Kurylo-Borowska, and L. C. Craig, *Biochemistry, 5,* 2153 (1966).

23. S. Wada and T. Toyota, *Biochem. Biophys. Res. Commun., 19,* 482 (1965).

25. E. E. Smissman, R. W. Sharpe, B. F. Aycock, E. E. van Tamelen, and W. H. Peterson, *J. Am. Chem. Soc., 75,* 2029 (1953).

26. H. E. Carter, W. R. Hearn, E. M. Lansford, Jr., A. C. Page, Jr., N. P. Salzman, D. Shapiro, and W. R. Taylor, *J. Am. Chem. Soc., 74,* 3704 (1952).

27. K. Nakanishi, T. Ito, and Y. Hirata, *J. Am. Chem. Soc., 76,* 2845 (1954).

28. H. Brockmann and H. Musso, *Ber., 88,* 648 (1955).

29. R. N. Costilow, O. M. Rochovansky, and H. A. Barker, *J. Biol. Chem., 241,* 1573 (1966).

30. T. C. Stadtman and P. Renz, *Federation Proc., 26,* 343 (1967).

31. R. C. Bray and T. C. Stadtman, *J. Biol. Chem., 243,* 381 (1968).

32. "University of Texas Publication No. 5109", University of Texas, Austin, Texas, 1951.

33. W. E. McGonigal, Ph.D. Thesis, Georgia Institute of Technology, 1965.

34. "Liquid Scinitllation Counting", Radiocarbon Laboratory, University of Illinois, Urbana Illinois, 1966, p. 42.

35. M. S. Patterson and R. C. Greene, *Anal. Chem., 37,* 854, (1965).

36. F. Eisenberg, "Liquid Scintillation Counting," Pergammon Press, New York, 1958, p. 123.

37. Paul D. Shaw, private communication, 1968.

38. E. N. Chappelle and J. M. Luck, *J. Biol. Chem.*, *229*, 171 (1957).

39. D. D. van Slyke, R. T. Dillon, D. A. MacFayden, and P. Hamilton, *J. Biol. Chem.*, *141*, 627 (1941).

40. D. E. Evans, A. C. dePaulet, C. W. Shoppe, and F. Winterniz, *J. Chem. Soc.*, *1957*, 1451.

41. S. Akabori, Y. Izumi, and T. Okuda, *Nippon Kagako Zusshi*, *77*, 490 (1956); see *Chem. Abstr.*, *52*, 8958 (1958).

42. S. Peterson and E. Tietze, *Ber.*, *90*, 920 (1957).

43. R. Kwok and P. Pranc, *J. Org. Chem.*, *32*, 738 (1967).

44. J. Thiele, *Ann.*, *270*, 1 (1892).

45. R. A. B. Bannard, A. A. Casselman, W. F. Cockburn, and G. M. Brown, *Can. J. Chem.*, *36*, 1541 (1958).

46. E. Hardegger and H. Ott, *Helv. Chim. Acta*, *38*, 312 (1955).

47. E. Fischer and R. Boehner, *Ber.*, *44*, 1335 (1911).

48. H. Meerwein, *Org. Syn.*, *46*, 113 (1966).

49. M. J. Buerger, "X-Ray Crystallography," John Wiley and Sons, Inc., New York, 1942, p. 512.

50. D. A. Carpenter, Ph.D. Thesis, Georgia Institute of Technology, 1968.

51. A. Zalkin, FORDAP Program.

52. W. R. Busing, K. O. Martin, and H. A. Levy, "A Fortran Crystallographic Least Squares Program," ORNL-TM-305, 1962.

53. J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, John Wiley and Sons, Inc., New York, 1961, pp. 1461-1477, and references cited therein.

54. Reference 18, pp. 928-940.

55. H. J. Vogel, *Federation Proc.*, *18*, 345 (1959).

56. Reference 18, pp. 668-669.

57. Reference 18, pp. 663-666.

58. T. C. Stadtman and L. Tsai, *Biochem. Biophys. Res. Commun., 28,* 920 (1967).

59. W. E. Streetman, private communications, 1968.

60. E. L. Jackson in "Organic Reactions," Roger Adams, ed., Vol. II, John Wiley and Sons, Inc., New York, 1944, p. 341.

61. W. Samki, *J. Biol. Chem., 187,* 369 (1950).

62. J. R. Dyer, "Methods of Biochemical Analysis," Vol. III, Interscience, Inc., New York, 1956, p. 133.

63. K. Schlossmann and F. Lynen, *Biochem. Z., 328,* 591 (1957).

64. K. Schlossmann, J. Brüggemann, and F. Lynen, *Biochem. Z., 336,* 258 (1962).

65. Reference 18, pp. 638-650.

66. H. Fasold, F. Turba, and W. Wirsching, *Biochem. Z., 335,* 86 (1961).

67. F. L. Scott, D. G. O'Donovan, and J. Reilly, *J. Am. Chem. Soc., 75,* 4053 (1953).

68. G. D. Buckley and T. J. Elliott, *J. Chem. Soc., 1947,* 1508.

69. F. Wessely, K. Schlogl, and G. Korger, *Monatsh., 83,* 678 (1952).

70. A. Étienne and Y. Corréia, *Comp. Rend. Acad. Sci., 259,* 2660 (1964).

71. R. E. Benson and T. L. Cairnes, *J. Am. Chem. Soc., 70,* 2115 (1948).

72. L. A. Pacquette, *J. Am. Chem. Soc., 86,* 4096 (1964).

73. R. A. B. Bannard, A. A. Casselman, W. F. Cockburn, and G. M. Brown, *Can. J. Chem., 36,* 1541 (1958).

74. W. R. Busing, K. O. Martin, and H. A. Levy, "A Fortran Crystallographic Function and Error Program," ORNL-TM-306 (1962).

75. A. C. McDonald and J. Trotter, *Acta Cryst., 18,* 243 (1965).

76. R. Destro, C. M. Gramaccioli, and M. Simonetta, *Chem. Commun., 1968,* 568.

77. B. W. Bycroft, D. Cameron, L. R. Croft, A. W. Johnson, and T. Webb, *Tetrahedron Letters, 1968,* 2929.

78. E. G. Miller, Jr., private communication, 1968.

79. J. C. Floyd, J. A. Bertrand, and J. R. Dyer, *Chem. Commun.*, in press.

80. J. R. Dyer, J. C. Floyd, P. D. Shaw, and R. DuBus, *Biochem. Biophys. Res. Commun.*, in press.

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