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7/25/68

THE CHEMISTRY OF VIOMYCIN

A THESIS

Presented to

The Faculty of the Graduate Division

by

William Edward Streetman

In Partial Fulfillment

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Doctor of Philosophy

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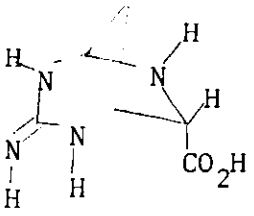
SUMMARY

Viomycin is a potent, broad spectrum antibiotic whose present use is in the treatment of tuberculosis cases that do not respond to other treatment. Viomycin was reported to be a peptide composed of serine, β-lysine, α,β-diaminopropionic acid (DAPA), viomycidine (I), urea, and carbon dioxide in the ratio 2:1:1:1:1:1. Research concerning the structure of viomycin has led to the proposed structures II and III from two different laboratories.



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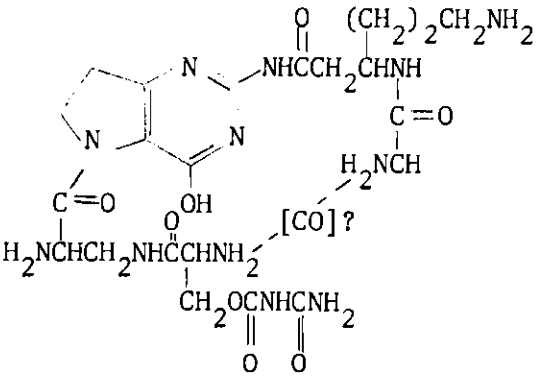

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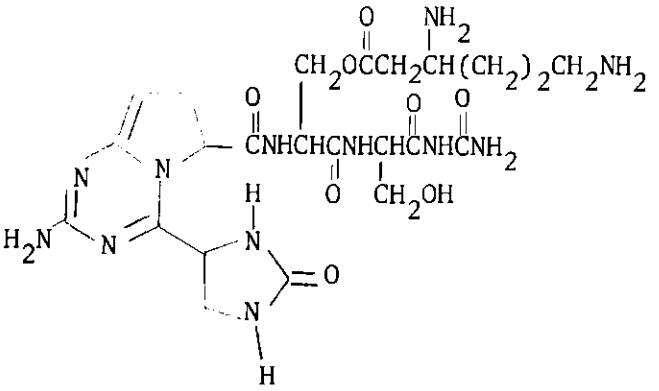


III

The purpose of the present research was to carry out further degradative studies leading to the correct structure of viomycin.

Early in this research it was learned that the commercially available viomycin was not homogenous; instead it was composed of a mixture of at least two components, both of which had antibiotic properties. Previous analyses of viomycin using paper chromatography in this laboratory had shown it to be chromatographically homogenous in every solvent system that had been used. Therefore a search was made for a method that could be used to analyse viomycin. Several methods were tried, but the only successful method was the use of buffered TLC plates. Analysis of viomycin by this method showed that it had at least two components.

Several attempts were made to separate these two components. Fractional crystallization of dye salts failed because viomycin was partially degraded in the formation of the dye salts. The two components were partially separated by means of gel filtration. The minor component, V_{min}, was eluted from the columns first; it comprised ca. 8-10 per cent of the mixture. The major component, V_{maj}, was obtained chromatographically homogenous from the last fractions to be eluted. The two components were then subjected to analysis in order to determine the difference between them. Their UV, NMR, and IR spectra did not differentiate between them. Minor differences were found in the NMR spectra, but these differences were not considered significant. Both V_{maj} and V_{min} were found by hydrolysis and amino acid analysis to have serine, DAPA, β-lysine, and viomycidine in the ratio 2:1:1:1. No urea was found in the hydrolysis product of V_{maj} or V_{min}. Thus the only difference found between V_{maj} and V_{min} was in their chromatographic behavior. Their behavior in the gel filtration

separation indicated that V_{min} has a greater molecular size or shape than V_{maj}.

Since no urea was found in the hydrolysis product of V_{maj} or V_{min}, it was concluded that a mutation had taken place in the microorganism that produces viomycin. The "old viomycin" contained urea and the "new viomycin" did not.

Johnson and coworkers later had proposed that the ultraviolted (UV) absorption of viomycin was associated with the urea unit. They had found that urea was released from viomycin during acid hydrolysis at the same rate that the chromophore was destroyed. They also concluded that the UV absorption was not due to any extent to the guanidine unit present in viomycin. Previous research in this laboratory had shown that the UV absorption was associated with a group with pKa 12.4, and the only group present in viomycin with that pKa is the guanidino group. Since the UV spectra of "old viomycin" and "new viomycin" were found to be identical in acidic and in basic solutions, it was concluded that they have the same chromophoric group. Also, the UV absorption is not due to the urea unit, since "new viomycin" does not contain a urea unit.

A basic compound was isolated from the hydrazinolysis product of viomycin that had absorption in the UV spectrum corresponding to the UV absorption of viomycin; this compound gave the guanidino compound viomycidine upon further hydrolysis.

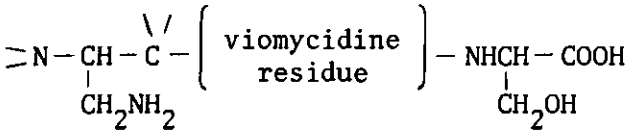
The elemental analysis of V_{maj} sulfate was found to correspond to the formula $C_{22}H_{35}N_{11}O_7 \cdot 3/2\ H_2SO_4 \cdot 5/2\ H_2O$. This formula has serine, β-lysine, DAPA, viomycidine, carbon dioxide, and ammonia in the ratio 2:1:1:1:1:1. The ammonia found in the acid hydrolysate of "old viomycin" was reported to be an artifact of the hydrolysis. Comparison of the a-

mounts of ammonia in the amino acid analyses of "old viomycin," V_{maj}, and V_{min} led to the conclusion that the ammonia in "new viomycin" hydrolysates was not an artifact.

Previously, few successful degradation methods had led to the isolation of sizeable peptide fragments from viomycin. It was found that hydrolysis of "new viomycin" in 0.01 N hydrochloric acid at 100° led to the formation of two products, both of which had UV absorption. In one component, the λ_{max} had been shifted to longer wavelength. The other component had the same UV spectrum as viomycin. No other degradation of the viomycin molecule was noted. A method was found to obtain these compounds in reasonably pure form.

Hydrolysis of viomycin in 6 N hydrochloric acid at room temperature led to the release of β-lysine and ca. one equivalent of serine. No carbon dioxide was produced; the UV chromophore was shifted to longer wavelength. A peptide was isolated from this hydrolysate that contained DAPA, viomycidine and serine. This peptide was named Peptide I. The UV spectrum of this material indicated that it might be a mixture of two very similar compounds with different UV spectra that were not readily seperable; the material appeared homogenous by TLC and electrophoresis. The major component had its UV absorption shifted to longer wavelength than the UV absorption of viomycin. It was thought that this shift might be due to a dehydration reaction that extended the conjugation of the chromophore.

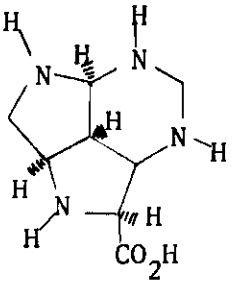
End group analysis of peptide I revealed that the carboxyl group of serine and the β-amino group of DAPA were free. Partial structure IV was assigned to Peptide I.



IV

Hydrolysis of viomycin with a strongly basic anion exchange resin gave peptide I, serine, and β-lysine as the major hydrolysis products.

Another guanidino compound, peptide III, was isolated from the acid hydrolysate of viomycin. It formed a crystalline dihydrobromide salt ($C_9H_{15}N_5O_3 \cdot 2$ HBr). It gave DAPA and viomycidine upon acid hydrolysis which showed that one nitrogen atom of peptide III was common to both hydrolysis products. A crystal of peptide III hydrobromide was found suitable for x-ray analysis; this analysis is being carried out. Preliminary results show that it has the same space group and cell dimensions as a compound isolated by Johnson and coworkers that they named viocidic acid. They reported its formula as $C_8H_{13}N_5O_2 \cdot 2$ Hbr$\cdot 3$ H_2O. They assigned structure V to it on the basis of an x-ray analysis. The analytical data of

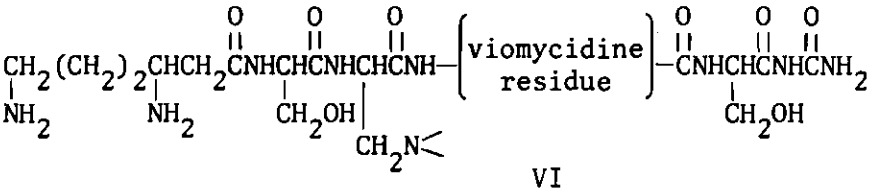


V

anhydrous peptide III and air dried peptide III did not agree very well with the formula $C_8H_{13}N_5O_2 \cdot 2$ HBr or its mono-, di, or tri-hydrates. It was concluded that there was some doubt whether the formula and structure

of peptide III were the same as those reported for viocidic acid and that this doubt should be resolved by the x-ray analysis of the structure of peptide III.

Very recently Kitagawa and coworkers reported that the peptide sequence of viomycin was VI.



VI

None of the data obtained in this research is in conflict with this proposed peptide sequence except that urea was not found in the viomycin used in this research.

CHAPTER I

INTRODUCTION

Isolation and Biological Studies

Viomycin is a potent tuberculostatic antibiotic whose toxic side effects make it presently useful only in those cases in which the patient does not respond to other treatment. The antibiotic was first reported in 1951 by workers in the laboratories of Charles Pfizer and Company (1) and Parke, Davis and Company (2). Later clinical studies showed that kidney damage, vestibular dysfunction, electrolyte imbalance, and hypersensitivity resulted from its extended use (3).

Since the structure of any potent antibiotic is useful in the study of the biological action of such compounds, the elucidation of the structure of viomycin has proceeded in several laboratories.

Properties of the Intact Molecule

Viomycin is a water soluble, optically active, strong organic base. It has pKa values* of 8.3, 10.3, and 12.2 in water due to the presence of two amino groups and a guanidino group (4). It forms a trihydrochloride salt, and its sulfate, picrate, and reinecate salts have been prepared. Viomycin sesquisulfate is an amorphous material that has a specific rotation of -39.8° in water and which decomposes at 252° (2).

---

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

Viomycin has been characterized by its nuclear magnetic resonance (NMR) and ultraviolet (UV) spectra. The NMR spectrum of viomycin sesquisulfate in deuterium oxide contains absorptions at τ 1.9-2.1, 5.7-6.4, 6.5-7.6 and 8.0-8.4 in the ratio 1:7.4:6.8:6.5, corresponding to the nonexchangeable protons (5). The UV spectrum of viomycin sesquisulfate contains a strong absorption whose position depends upon the pH of the solution. The λ_{max} occurs at 268.5 mμ (ε, 23,000) in 0.1 N aqueous hydrochloric acid and 282.5 mμ (ε, 14,500) in 0.1 N aqueous sodium hydroxide (4). Studies of the variation of the position of absorption with pH showed two isosbestic points at 235 mμ and 281 mμ. Analysis of these data indicated that one dissociating group, pKa 12.4, was involved in the chromophore. The group in viomycin with pKa 12.4 is the guanidino group (4,6).

The exact molecular formula of viomycin is not as yet known. Formulas that have been suggested are $C_{18}H_{31-33}N_9O_8$ from analysis of viomycin salts (3,7); $C_{25}H_{44}N_{12}O_{11}$ from analyses and determination of the molecular weight as 625 by diaphragm-cell diffusion (4); $C_{23}H_{41}N_{13}O_9$ from degradative studies (5,8) and $C_{23}H_{38}N_{12}O_9$ from further degradative studies (9). A comparison of the agreement of these formulas with reported analyses of viomycin sesquisulfate is given in Table 1. The differences in reported analyses are at least partially due to the difficulty in obtaining either anhydrous viomycin or a stoichiometric hydrate from the amorphous sulfate salt.

Table 1. Comparison of Analytical Data for Viomycin Sesquisulfate

Formula per cent	C	H	N	S	ratio C/N
$C_{25}H_{44}N_{12}O_{11}\cdot 3/2\ H_2SO_4$	35.93	5.67	20.11	5.75	1.79
$C_{23}H_{41}N_{13}O_9\cdot\ 3/2\ H_2SO_4$	34.92	5.61	23.03	6.08	1.52
$C_{23}H_{38}N_{12}O_9\cdot 3/2\ H_2SO_4$	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)	34.22[b]	5.73[b]	21.85[b]	4.73[b]	1.56
Reported (5)	35.01	6.19	21.01	5.73	1.67
Reported (7)	35.89	5.52	21.15	5.79	1.70
Average	35.71	5.83	21.19	5.51	1.69

[a] determined as sulfate

[b] not corrected for residue after ignition (1.25%)

Complete Degradation of Viomycin

A positive biuret test and resistance to mild acid hydrolysis suggested that viomycin is a peptide. This was confirmed by the isolation of the amino acids L-Serine, L-3,6-diaminohexanoic acid (β-lysine), L-2,3-diaminopropionic acid (DAPA), and 2,4,6-triaza-3-iminobicyclo[3.2.1]-octane-7-carboxylic acid (viomycidine) (I) after vigorous hydrolysis with 6 N aqueous hydrochloric acid (1,2,4,7,10,11). Urea, carbon dioxide, ammonia, and small amounts of guanidine-containing compounds were also released in this hydrolysis. The UV chromophore was also destroyed. The relative amounts of the compounds released after hydrolysis of viomycin with 6 N aqueous hydrochloric acid for six and sixteen hours are shown in Table 2. Serine, urea, β-lysine, DAPA, and viomycidine were reported to occur in the ratio 2:1:1:1:1. Ammonia released during the hydrolysis was probably due to partial degradation of urea and some of the amino acids (9). The structures of serine, DAPA, and β-lysine were determined by comparison with the known compounds (4,7).

The major component of the mixture of guanidino compounds has been named viomycidine (8). It was an optically active base (pKa's 2.8, 5.87, 13.9 in 66 per cent N,N-dimethylformamide (DMF) and 5.50, 12.6 in water) (10). It formed crystalline monohydrochloride and monohydrobromide salts. Positive reactions with Weber and Sakaguchi reagents indicated a mono-substituted guanidino group. A positive reaction with ninhydrin characteristic of amino acids was observed (4,10). Viomycidine formed a mono-2,4-dinitrophenyl derivative. When treated with aqueous ethanolic acetic anhydride, viomycidine formed a mono-acetate (8,9). Hydrolysis of viomycidine with barium hydroxide at 95°C for 77 hours gave ammonia, carbon

Table 2. Compounds Released in the Acid Hydrolysis of Viomycin[a]

Compound	6 Hours (7)	16 Hours (4)	16 Hours (13)
Carbon dioxide	0.50	0.88	-
Ammonia	0.70[b]	0.92[b]	0.84[f]
Urea	0.74[d]	0.40[c]	0.0
DAPA	0.42[e]	0.14[e]	0.95[f]
β-lysine	0.90[e]	0.44[e]	1.07[f]
Serine	-	1.30[e]	1.79[f]
Viomycidine	0.21[e]	0.16[e]	0.42[f]

[a]All values expressed as moles per mole of viomycin (assumed molecular weight 688).

[b]Ammonia was determined as volatile base by aeration from basic solution into boric acid.

[c]Urea was determined by conversion to ammonia with urease followed by determination of the ammonia.

[d]Urea was determined by the biacetylmonoxime method of Ormsby (12).

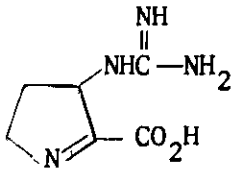
[e]The amounts of the amino acids were determined by isolation.

[f]The amounts of the amino acids were determined by large scale isolation using a long Dowex 50 (H+) cation exchange column (13).

dioxide and pyrrole-2-carboxylic acid (9). Hydrolysis of viomycidine with hot, concentrated sodium hydroxide gave 2-aminopyrimidine, glycine, and pyrrole-2-carboxylic acid (8,10).

The NMR spectrum of viomycidine in deuterium oxide revealed absorption at τ 7.43 (2H, triplet, J=2.1 cps), 5.38 (2H, multiplet), and 4.37 (1H, triplet, J-2.2 cps). These corresponded to the nonexchangeable protons. The NMR spectrum of viomycidine in trifluoroacetic acid (TFA) revealed other absorptions at τ 3.0 (1H, singlet), 1.98 (1H, singlet), and 1.48 (1H, singlet) (8).

Several structures have been proposed for viomycidine. An early structure proposed by Dyer, Hayes, and Miller (14), structure II, was incorporated into structure III, proposed by Johnson as the structure of the viomycidine moiety in viomycin itself (15). However, racemic viomycidine would be expected from the acid hydrolysis of viomycin if this structure were correct. When viomycidine was treated with hot acetic anhydride in pyridine followed by ozonolysis with oxidative workup and acid hydrolysis, di-aspartic acid was isolated. This was explained by structure IV proposed at this point by Dyer as a revised structure for viomy-



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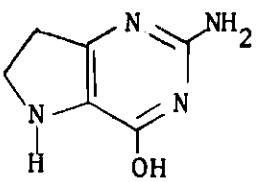

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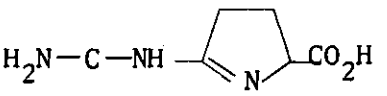
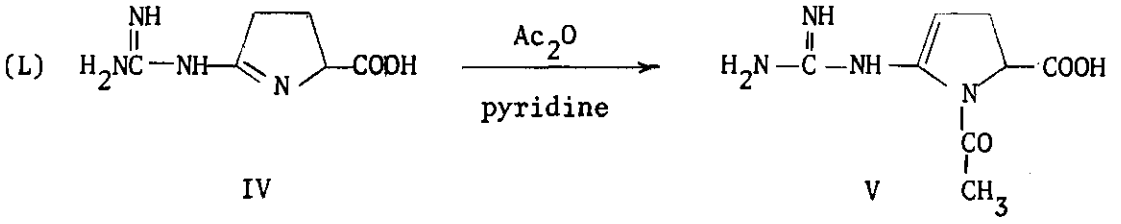


IV

cidine (16). The N-acetyl derivative (V) of IV was formed with a shift of the double bond. The double bond was cleaved by ozone; oxidative work-up followed by acid hydrolysis produced aspartic acid. The asymmetric center was racemized at some stage, perhaps under the basic conditions of acetylation. This degradation is shown in reaction 1.



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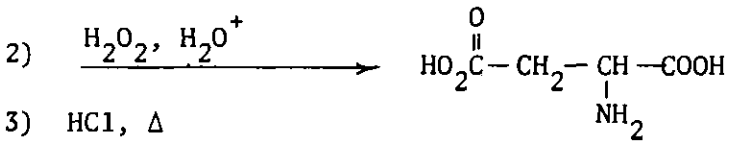
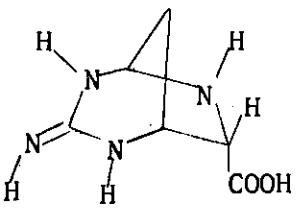


dl-aspartic

This structure was disproved when an X-ray diffraction analysis of viomycidine hydrobromide revealed that, as recently reported (17), 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid (I) is the correct structure of viomycidine (11).



Partial Degradation of Viomycin

When viomycin was allowed to react with 2,4-dinitrofluorobenzene (DNFB), bis-(2,4-dinitrophenyl)viomycin (bis-DNP-viomycin) was formed. Isolation of bis-DNP-β-lysine from the acid hydrolysate of this material showed that the two free amino groups of viomycin are the amino groups of β-lysine (5,8). Hydrazinolysis of viomycin gave no free amino acids; this indicated that no free amino acid carboxyl group existed in viomycin (5).

Viomycin gave urea, ammonia, carbon dioxide, L-DAPA, L-serine, L-β-lysine, dl-alanine, and pyrrole-2-carboxylic acid upon treatment with 0.5 N aqueous sodium hydroxide for three days (4,9).

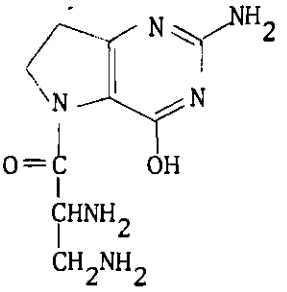
Viomycin was oxidized by treatment with aqueous potassium permanganate, bromine water, or ozone; the UV chromophore was destroyed. No difference was noted between the composition of the oxidation product acid hydrolysate and the composition of the usual viomycin acid hydrolysate (4,5).

Viomycin gave urea, serine, and β-lysine after one day when it was treated with 6 N aqueous hydrochloric acid at room temperature. After eleven days, a complex pattern of ninhydrin positive spots appeared. No viomycidine or DAPA was released (5).

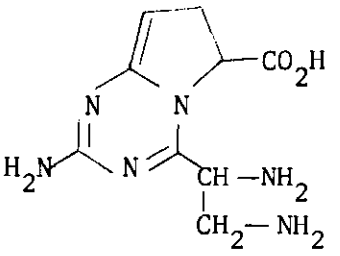
Viomycin was degraded by 0.1 N aqueous hydrochloric acid at 95°C for six hours to urea and desureaviomycin (4,5). End group analysis of desureaviomycin indicated that a serine carboxyl group and both amino groups of β-lysine were free. Desureaviomycin was degraded by carboxypeptidase to serine and viomycinic acid. End group analysis of viomycinic

acid indicated that a serine carboxyl group and both amino groups of β-lysine were free. Neither desureaviomycin nor viomycinic acid contained the UV chromophore of viomycin (5). From the data on desureaviomycin and viomycinic acid, it was concluded that a serylserylurea fragment existed in viomycin with the urea as a terminal group. Also, it was concluded that β-lysine is attached to the remainder of the molecule through its carboxyl group (5).

In 1964, Johnson reported the isolation of a dipeptide, DAPAylviomycidine, which contained the chromophore of viomycin (15). He postulated structure VI for this compound using Dyer's early structure for viomycidine (II). Later, Dyer proposed structure VII for this dipeptide, using the revised structure of viomycidine (IV) (16). Johnson also reported



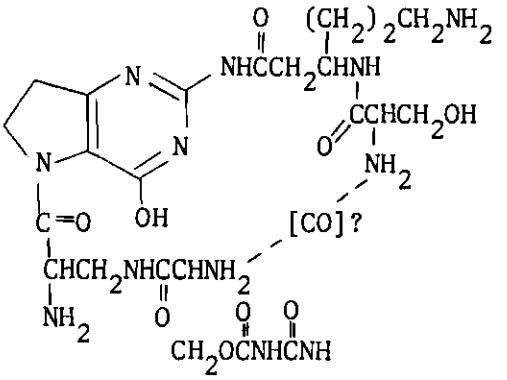
VI



VII

the isolation of the peptide seryl-β-lysine (ε-NH_2 free) from acid and base hydrolysis of viomycin. He found that ε-mono-DNP-β-lysine and α-mono-DNP-DAPA were present in the hydrolysate of bis-DNP-viomycin. Another peptide gave equimolar amounts of β-lysine and viomycidine upon

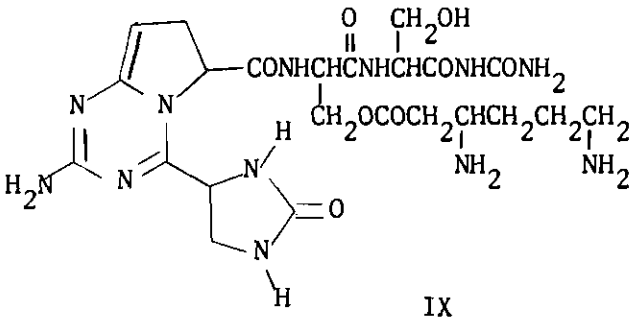
further hydrolysis. A fourth peptide was formulated as seryl-β-lysyl-(ε-NH_2 free)-viomycidine from end group analysis data. Since compounds giving urea and serine, and urea, serine and DAPA on further hydrolysis were isolated from the permanganate oxidation product of viomycin, it was proposed that the second serine molecule was bound to urea through its hydroxyl group and to the β-amino group of DAPA through its carboxyl group. Structure VIII was then proposed as the structure of viomycin, with the carbon dioxide precursor a ureide linkage between the amino groups of the serine molecules.



VIII

Structure VIII was contradicted by the revised structure for viomycidine and the isolation of desureaviomycin and viomycinic acid. Bis-DNP-β-lysine could not be obtained from this structure. No proton would be expected to have an absorption in the NMR spectrum at τ 2.0, as observed for viomycin. Therefore Dyer proposed structure IX in 1965 as the

structure of viomycin (19).

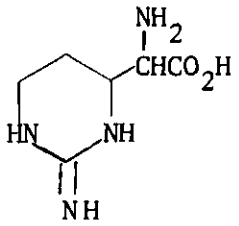


IX

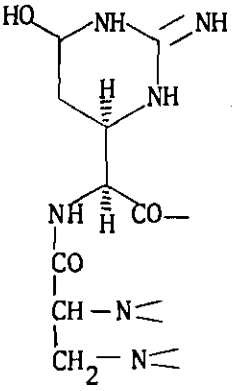
The purpose of the present research was to determine the correct structure for viomycin or to determine as many of the structural features of viomycin as possible. It was known that the commercially available viomycin was not homogenous (20); a further purpose in this research was to obtain pure viomycin and repeat some of the previous work using the pure material.

Very recently, Johnson has reported further degradative studies on viomycin (21). Degradation of viomycin with 0.1 N aqueous sodium hydroxide gave a high yield of 2-aminopyrimidine and a dipeptide DAPAyl-glycine. Acid hydrolysis of the hydrogenation product of viomycin gave capreomycidine (X), a degradative product of the capreomycin group of antibiotics (22,23). Structure XI was proposed as the representation of their dipeptide DAPAylviomycidine in the intact molecule of viomycin. The structure of viocidic acid (XII), a degradation product of viomycin, was determined by an X-ray diffraction analysis. It was suggested that, since destruction of the chromophore of viomycin occurred with the release of urea, urea was a part of the chromophore. Furthermore, they suggested

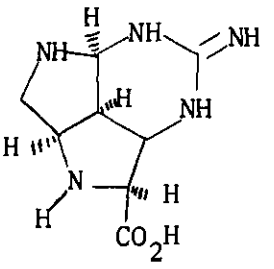
that the guanidino group was unlikely to be a part of the chromophore.



X



XI



XII

Kitagawa and coworkers isolated a series of peptides through partial hydrolysis of viomycin (24). These peptides were characterized chromatographically, chemically, and through their UV spectra and optical rotations as shown in Table 3. From these data, the amino acid sequence of peptide C is serylDAPAyl(free-β-NH_2)viomycidine; peptide B is β-lysylserylDAPAyl(free-β-NH_2)viomycidylserine. Peptides A_I, A_{II} and A_{III} are differentiated by chromatography and electrophoresis; their peptide sequence is β-lysylserylDAPAylviomycidylserine where no amino group of DAPA is free. Since they found that peptide A_{III} and urea were the products of mild acid hydrolysis of viomycin, they suggested that the amino acid sequence of viomycin is β-lysylserylDAPAyl(no free NH_2)viomycidylserylurea (XIII).

Table 3. Peptides Isolated from Partial Hydrolysates of Viomycin (24)

Peptide	UV H_2O λ_{max}, log ε	Sakaguchi Test	R[a]	R_F[b]	$[\alpha]_D$	Components[c]	Carboxyl Terminal Acid[d]	Amine Terminal Acid[e]
A_I	269.5, 3.21	+	0.98	0.25	-43°	ser,β-lys,DAPA viomycidine, 2:1:1:1	ser	β,ε-NH_2 of β-lys
A_{II}	285, 3.93	+	0.88	0.11	-49.5°	ser,β-lys,DAPA viomycidine,2:1:1:1	ser	β,ε-NH_2 of β-lys
A_{III}	272, 3.95	+	0.9	0	-37°	ser,β-lys,DAPA, viomycidine,2:1:1:1	ser	β,ε-NH_2 of β-lys
B	268, 4.12	+	0.95	0.5	-38°	ser β-lys,DAPA viomycidine, 2:1:1:1	ser	β,ε-NH_2 of β-lys β-NH_2 of DAPA
C	...	+	0.8	0.6	-2°	ser, DAPA, viomycidine, 1:1:1	viomycidine	β-NH_2 of DAPA

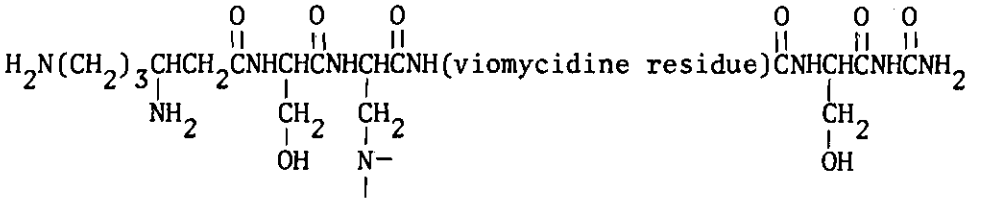
[a]The R values are determined from electrophoresis; R=mobility of compound/mobility of viomycin.

[b]The R_F values are for paper chromatography in the system t-butyl alcohol:acetic acid:water::2:1:1.

[c]The components were determined by an amino acid analyser.

[d]The carboxyl terminal acid was determined by hydrzainolysis.

[e]The amine acid was determined by analysis of the corresponding DNP derivative.


H2N(CH2)3CHCH2CNHCHCNHCHCNH(viomycidine residue)CNHCHCNHCNH2
NH2
CH2
OH
CH2
N–
CH2
OH

XIII

CHAPTER II

EXPERIMENTAL

Apparatus and Techniques

Ion Exchange Materials

Ion exchange resins were used for neutralization of solutions, change of the anion in amino acid salts, and for separation of the amino acids and peptides. Resins used were the weakly basic anion exchange resin Amberlite IR-45, the strongly basic anion exchange resin Amberlite IRC-50 (Mallinckrodt Chemical Works). These resins were used as described previously (5,8,9,10). In addition, the strongly basic anion exchange resin Dowex-1X8 (J. T. Baker Chemical Co.), and the strongly acidic cation exchange resin Dowex-50X8, and the weakly acidic anion exchange resin Sephadex CM C-25 (Pharmacia Fine Chemicals, Inc.) were used. The Dowex-1X8 was regenerated during three cycles between the acid and base forms using 4 N acid and base. The resin was washed with distilled water until the supernatent liquid was at pH 10 before changing it to the acid form, and the acid form was washed with distilled water until the supernatant liquid was at pH 2 before changing it back to the base form. In the final cycle, the resin was left in the basic form and used for basic hydrolysis of viomycin. The Dowex-50X8 is a chromatography grade resin used in large scale separations of the viomycin hydrolysis fragments. It was prepared for use by allowing it to stand in 6 N aqueous hydrochloric acid for at least 30 min., and subsequently washing it with distilled water

until the supernatant liquid was at pH 5-6. The Sephadex CM C-25 was first hydrated in water. After packing it as a slurry in a column, the ionic form was made acidic or neutral using 1 N aqueous hydrochloric acid or 1 N aqueous sodium hydroxide. Then the column was eluted with distilled water until the effluent was at pH 7, at which point the column was ready for use.

Qualitative Analysis

Apparatus and techniques used in paper chromatography were the same as those described previously (5,8,9,10). Thin layer chromatography (TLC) was routinely used for qualitative analysis of amino acid mixtures. TLC plates were prepared according to Stahl (25) using silica gel H and silica gel HF_{254} (Brinkman Instruments Incorporated). In addition, buffered TLC plates (CB plates) were prepared using a 0.05 M pH 6 citrate buffer instead of water as the slurrying solvent. The plates were activated by heating for one to twelve hours after air drying and were stored over anhydrous silica gel until used. Developing solvents commonly used in paper chromatography and TLC were abbreviated as follows: CB, 30% pH 6 citrate buffer in methanol; BAW, t-butyl alcohol:acetic acid:water:: 2:1:1; BW, 1-butanol saturated with water, BAAmW, t-butyl alcohol:acetic acid:five per cent aqueous ammonia:water::5:4:3:1; and BAcAAmW, t-butyl alcohol:acetone:acetic acid:five per cent aqueous ammonia:water::5:4:4: 3:1. Dimethylsulfoxide (DMSO) and N,N-dimethylformamide (DMF) were used in various combinations with other solvents. Ninhydrin, Weber, and p-dimethylaminobenzaldehyde (pDMAB) spray reagents were used as described previously (26) for the visualization of spots corresponding respectively to amino acids, guanidine containing compounds, and urea in paper chroma-

tography, TLC, and electrophoresis. Iodine vapor and UV lamps were also used for visualization of spots on TLC plates.

A Beckman Model R electrophoresis apparatus was used (27). The buffer systems most frequently used were a pH 4.0 sodium acetate buffer and a pH 9.4 ammonium formate buffer. Analyses were made using Beckman No. 320046 paper strips, and a constant current of 5-25 milliamperes was usually applied for times of 1.5-12 hrs. Usually, standard samples were analysed concurrently with the unknown samples. The characteristic mobilities of the compounds were recorded as the distance the compound moved from the origin, in millimeters, with the buffer system, current and time used. Relative intensities and colors of the bands were abbreviated as follows: heavy-h, medium-m, faint-f, purple-pu, blue-bl, brown-br, red-r, pink-p, and grey-g.

Chromatographic Separations

Partially deactivated carbon columns (10) and powdered cellulose columns (9) were prepared and used as described previously.

Mixtures of diatomaceous earth and silicic acid were used as described previously for purification of DNP-amino acids and DNP-peptides (28,29). Gel filtration separations were carried out with Bio Gel P-2 (California Biochemical Company) and Sephadex G-10 and G-15 (Pharmacia Fine Chemicals, Inc.). The material was allowed to stand for two hours in an excess of 0.01 N aqueous formic acid; it was then ready to pour as a slurry into the column. After gravity packing, the column was ready for use; it could be reused after washing it with 10-12 column volumes of 0.01 N formic acid.

Instruments

Varian A-60A and A-60D 60 megacycle NMR instruments were used to obtain NMR spectra with tetramethylsilane (TMS) or sodium 2,2-dimethyl-2-silapentanesulfonate (DSS) as internal standards. A Cary Model 14 recording spectrophotometer was used for UV spectra, and Perkin Elmer Models 137 and 337 infrared (IR) spectrometers were used for IR spectra.

Automatic fraction collectors used were a GM Instrument Company, Inc. Model VE-2002-B-24 and a Research Specialties Model 1205. Ozone for ozonolyses was produced in a Welsbach model T-23 ozone generator. Optical rotations were taken with a Bellingham and Stanley Model 397619 polarimeter. Rinco rotary evaporators were used for removal of solvent from solutions. All melting points were determined using a Köfler micro hot stage.

Microanalyses were performed by A. Bernhardt Microanalytical Laboratories (Müllheim, West Germany).

Purification and Characterization of Commerical Viomycin

Qualitative Analysis of Commercial Viomycin

A solution of commercial viomycin (Parke, Davis and Company, lot No. X4436) was analysed by TLC and paper chromatography and was found to have components with the R_F values given in Table 4.

Commercial viomycin was also analysed by electrophoresis. One band was observed at -55 + 8 mm with a constant current of five milliamperes for two hours in the pH 4.0 buffer system, and one band at -82 ± 7 mm with a constant current of five milliamperes for three hours in the pH 9.4 system.

Table 4. R_F Values of Commerical Viomycin in Various Systems.

Medium	Solvent	Visualization	R_F Values
Whatman No. 1 paper	BAW	ninhydrin	0.0
Silica gel HF_{254} TLC plates	BAW, BW, H_2O	ninhydrin, UV light	0.0
CB TLC plates	CB	ninhydrin, UV light	0.0, 0.22

Attempted Purification of Viomycin By Preparation of Dye Salts.

Orange II, an azo dye (Fisher Scientific Co.), was converted to the acid form using a method for the conversion of sodium p-hydroxyazobenzene-p'-sulfonate to its acid form (30). A saturated solution of the free acid of Orange II was added to a solution of 1.0 g of commercial viomycin sulfate (1.27 mmoles) in 15 ml of water until no further precipitation occurred. The dark red precipitate that formed immediately turned into an oil. After trituration with methanol, the oil solidified; 1.8 g of viomycin • 3 Orange II (1.08 mmoles, 85 per cent) was recovered. A solution of 0.50 g of viomycin • 3 Orange II in 100 ml of water was neutralized with 75 ml of IR-45 (OH^-). The mixture was filtered and washed with 100 ml of water. The filtrate and washings were combined and passed over a column of 21 ml of IR-45 ($SO_4^=$). The solution was evaporated in vacuo to a small volume and lyopholized. The product was 0.329 g of a white amorphous solid. This material gave one spot at R_F 0 with a streak to R_F 0.17 on CB TLC plates in CB. The UV spectrum of this material had a

very broad $\lambda_{max}^{H_2O}$ at 285 mμ ($E_{1cm}^{1\%}$, 120).

Fifty milliliters of a ten per cent solution of p-hydroxyazobenzene-p-'-sulfonic acid (pHABS) was added to a solution of 1.0 g (1.27 mmoles) of viomycin sulfate. A gold precipitate formed when this solution was added drop by drop to 600 ml of acetone with stirring. The dried precipitate weighed 1.3 g (63 per cent). A solution of 0.50 g of viomycin ·3 pHABS (0.31 mmoles) in 30 ml of water was neutralized with 75 ml of IR 45(OH^-). The mixture was filtered and the resin was washed with three 30-ml portions of water. The filtrate and washings were combined and passed over a column of 20 ml of IR-45($SO_4^=$). The eluate was evaporated in vacuo at 50° to a few milliliters and lyopholized to give 0.396 g of a creamy white solid. A spot at R_F 0.0 and a very faint spot at R_F 0.22 were observed when this material was analysed by TLC on CB plates in CB. The UV spectrum of this material had a $\lambda_{max}^{H_2O}$ at 270 mμ ($E_{1cm}^{1\%}$, 179) with a shoulder at 300 mμ.

Gel Filtration of Viomycin Using Bio Gel P-2

Seven hundred grams of Bio Gel P-2 was stirred for 3-4 hr with 0.01 N aqueous formic acid. The hydrated gel filled a column 219 cm high x 3.4 cm I.D. to a height of 208 cm (1890 ml). The column was washed with ca. 2.5 l of 0.01 N formic acid during the packing process. The stoichiometric viomycin sulfate was prepared from the commercial viomycin as reported previously (5). A sample of 4.980 g viomycin sulfate in 10 ml of water was applied to the top of the column; it was washed on with three portions of five milliliters each of 0.01 N formic acid. The material was eluted from the column by 0.01 N formic acid. A total of 120 fractions of 15 ml each were collected at a flow rate of 1.15 ml/min.

Fractions 55-115 gave positive ninhydrin tests; these fractions were lyopholized, weighed, and analysed by TLC. Figure 1 is a graph of weight/fraction *vs* fraction number. Fractions 55-85 contained 0.088 g of material which had only one spot at R_F 0 on CB plates with CB as solvent. Fractions 86-95 contained 1.451 g of a mixture which had two spots, R_F 0.0 and R_F 0.22 in the same TLC system. Fractions 95-110 contained 2.703 g of material with R_F 0.22. The material with R_F 0.0 was the minor component; it will be referred to as V_{min}. The material with R_F 0.22 is the major component; it will be referred to as V_{maj}. Total recovery was 4.381 g (88 per cent).

Gel Filtration of Viomycin Using Sephadex G-15

A column 220 cm high x 3.4 cm I.D. was packed to a height of 210 cm with 2000 ml of Sephadex G-15 which had been hydrated with 0.01 *N* formic acid. In a typical run, a sample of 6.61 g of viomycin sulfate in two milliliters of water was applied at the top of the column and washed on with three portions of two milliliters each of 0.01 *N* formic acid. The material was eluted with 0.01 *N* formic acid; 120 fractions of 20 ml each were collected at a flow rate of 2.0 ml/min. Fractions 44-64 gave a purple color when two drops from each sample was heated with one milliliter of ninhydrin reagent. These fractions were lyopholized, weighed, and analysed by TLC. Figure 2 is a tracing of the TLC pattern obtained with CB plates and CB solvent; Figure 3 is a graph of fraction weight *vs* fraction number. Fractions 44-47 contained 0.145 g of pure V_{min}. Fractions 51-64 contained 5.122 g of pure V_{maj}. Fractions 48-50 contained 1.132 g of the mixture of V_{maj} and V_{min}. The total amount recovered was 6.41 g (97 per cent). When 1.00 g of this mixture was rechroma-

tographed using a much longer column (1350 ml Sephadex G-15 in a column 550 cm high x 1.9 cm I.D.), the second weight curve (dashed line) in Figure 3 was obtained. Fractions of 14 ml at a flow rate of 1.4 ml/min were taken. Pure V_{min} was in fractions 39-41 (0.042 g); pure V_{maj} was in fractions 43-50 (0.814 g), and the mixture was in fraction 42 (0.066 g). The material recovered was 92 per cent of the starting material.

Attempted Crystallization of V_{maj}

An attempt was made to crystallize the free base of V_{maj} by the method of Wild, which was successfully used to crystallize the free base of caprecomycin, an antibiotic that is similar to viomycin (31). A sample of 0.221 g of V_{maj} sulfate was applied to a column of ten milliliters of Dowex-1 X8 (OH^-) anion exchange resin; the column was eluted with 250 ml of freshly boiled distilled water. This solution was evaporated to dryness *in vacuo* giving a white powder which did not completely redissolve in five milliliters of distilled water. The insoluble material was separated by centrifugation. The supernatant solution was decanted and ten milliliters of redistilled methanol were added; 1-propanol was then added drop by drop until the solution became slightly cloudy. A gel-like precipitate formed upon cooling, and it was separated by centrifugation; it did not completely redissolve in water. Another attempt was made to crystallize the water soluble material; more water-insoluble material was obtained. No crystalline material was obtained.

Characterization of V_{maj}

Spectral Properties of V_{maj}

A sample of V_{maj} sulfate (28.9 mg, 0.0361 mmoles) that had been

dried for 72 hours at 80° under high vacuum was dissolved in water. The solution was diluted to 100 ml. Ten milliliter aliquots from this solution were diluted to 100 ml with 1 N hydrochloric acid, with water, and with 1 N sodium hydroxide. The UV spectra of these solutions had λ_{max}^{HCl} at 268.8 mμ (ε, 25,700), $\lambda_{max}^{H_2O}$ at 268.4 mμ (ε, 24,700), λ_{max}^{NaOH} at 290.5 mμ (ε, 17,500). The molar extinction coefficients were calculated using 800 as the molecular weight of V_{maj} sulfate. The NMR spectrum of a 17 per cent solution of V_{maj} (dried to constant weight for three days at 80°C under high vacuum) in D_2O was taken with DSS as internal standard. Absorptions centered around τ 1.9 (doublet, 3.6 (doublet), ca. 5 (multiplet), ca. 6.9 (multiplet), ca. 8.2 (multiplet) had relative intensities (by planimeter) of 1:0.6:20-22:9:7-8:8, making a total of 45-48 protons. The IR spectrum of V_{maj} sulfate was taken as a nujol null; this spectrum was essentially identical with that of commercial viomycin sulfate (5).

Elemental Analysis of V_{maj}

A sample of V_{maj} sulfate was dissolved in water. This solution was centrifuged and the supernatant solution was lyopholized. The white V_{maj} sulfate was then dried to constant weight after three days at 80°C under high vacuum. It lost 12 per cent of its weight during the drying process. This sample of V_{maj} sulfate gave an analysis of per cent C, 34.56; H, 5.58; N, 20.43; O, 32.84; and S, 6.50.

Amino Acid Analysis of V_{maj}

A sample of V_{maj} sulfate was submitted for amino acid analysis.* The sample was hydrolyzed in 6 N hydrochloric acid in a sealed tube at

*We wish to thank Eli Lilly and Company for doing amino acid analyses for us. A Moore-Stein amino acid analyser modified according to Hubbard (32) was used.

105° for 21-24 hr. The results, given as μmoles/mg sample were: serine 1.74, β-lysine 2.05, DAPA 1.02, viomycidine 0.880, ammonia 1.79, glycine 0.155, alanine 0.051, and a trace of valine. Several unknown peaks were also present.

Complete Hydrolysis of V_{maj}

A solution of 0.098 g of V_{maj} sulfate in two milliliters of 6 N hydrochloric acid was heated under reflux at 97° for 20 hr. The product, a red solution, was evaporated in vacuo to dryness; five milliliters of water was added and the solution was evaporated to a red-brown oil. This oil was dissolved in one milliliter of water and the solution was analysed by electrophoresis. Mobilities found for the components of this hydrolysate and for standard solutions are shown in Table 5. The solution

Table 5. Electrophoresis Mobilities of the Acid Hydrolysate of V_{maj} sulfate.

Hydrolysate	β-lysine	peptide III	viomycidine	DAPA	serine
-50±3 pu.,h.	-49±4 pu.,h.				
-26±2 br.,h.		-27±3 br.,h.			
-21±3 pu.,f.					
-6±3 pu.,h.			-8±4 pu.,h.		
				+3±3 pu.,h.	
+9±7 pu.,h.					
+28±4 pu.,f.					+13±4 pu.,h.

[a]Conditions: pH 9.4, 25 ma for two hours, spray-ninhydrin.

was analysed by TLC. The hydrolysate solution and a urea solution were chromatographed on the same TLC plate in BAW. The plate was dried and sprayed with pDMAB reagent. A faint orange-yellow spot appeared at R_F 0.77 in the hydrolysate chromatogram; a bright yellow spot at R_F 0.83 was observed in the urea chromatogram.

Hydrazinolysis of V_{maj}

A sample of 0.125 g (0.15 mmoles) of V_{maj} sulfate that had been dried for 29 hr at 100° was dissolved in one milliliter of anhydrous hydrazine and heated at 100° in a sealed tube for 20 hr. The tube was cooled and opened; the light red solution was evaporated to dryness over concentrated sulfuric acid *in vacuo* to give a red gum. This gum was dissolved in one milliliter of water; two milliliters of pH 7 buffer and one milliliter of redistilled benzaldehyde were added. This mixture was allowed to stand overnight with occasional shaking. After extraction with two 25-ml portions of ether, the aqueous layer was evaported *in vacuo* to 0.5 ml; this was analysed by TLC and electrophoresis. A ninhydrin and Weber positive spot was observed at R_F 0.56 (CB plates, CB solvent), R_F 0.67 (BAW solvent), and R_F 0.64 (BAcAAmW solvent). Faint spots with lower R_F values were also observed. The R_F values of serine in these systems were respectively 0.57, 0.64, and 0.68. No spot gave a color with the pDMAB spray reagent. One heavy band at -78 ±6mm (pH 9.4, 25 ma for 3.5 hr) was observed; serine had a band at +12 ±3mm. No band was observed in the hydrazinolysate electrophoresis pattern that corresponded to serine even when second and third samples were run at two and four times the original concentration. A faint band was observed at -93 ±5mm. Besides the ninhydrin positive bands, a band that was brown without spray-

ing was observed at R_F 0.70, 0.81, and 0.85 in the three TLC systems and at -19 ±4mm in the electrophoresis system. Preparative electrophoresis was carried out on this sample using a sheet of Whatman No. 17 chromatography paper 12 x 12 in; the electrophoresis conditions were 45 ma for three hours in the pH 9.4 buffer. Narrow strips were then cut from each side of the sheet and from the middle; these strips were sprayed with ninhydrin. The desired ninhydrin positive band appeared at -46 ±11mm. This band was cut out of the paper and extracted with two 25-ml portions of boiling water. The aqueous extract was evaporated *in vacuo* at 70°C to give 0.015 g of a brown gum; then UV spectrum of this gum had a $\lambda_{max}^{H_2O}$ at 282 mμ ($E_{1cm}^{1\%}$, 103). This sample was evaporated to dryness again and dissolved in 0.4 ml of 6 N hydrochloric acid. This solution was heated for 22 hr. at 110°C. The hydrolysate had bands at -66 ±5mm, pu, m and -21 ±5mm, pu, m (pH 9.4, 25 ma for 5.7 hr). At the same time serine, DAPA, viomycidine and β-lysine had mobilities of +50 ± 8, +19 ± 6, -19 ± 5, and -60 ± 2 mm. This hydrolysate had two overlapping purple spots at R_F values of *ca*. 0.40 and 0.53 (TLC with CB solvent); serine, viomycidine, DAPA and β-lysine had R_F values of 0.64, 0.51 and 0.36, 0.45 and 0.34 respectively on the same plate.

A sample of 0.148 g (0.019 mmoles) of V_{maj} sulfate in two milliliters of anhydrous hydrazine in a sealed tube was heated at 100° for 18 hr; the tube was opened and evaporated *in vacuo* over concentrated sulfuric acid to a red gum that was then analysed by TLC and electrophoresis. Four ninhydrin positive spots, R_F 0.0, 0.14, 0.27 and 0.45, were observed with BAW. The spots at R_F 0.27 and 0.45 were also Weber positive. Bands at +32 ±2mm (brown without spray); +11 ±3 mm, pu, f; +2 ±2 mm, pu, f; -10

±3 mm, br, m; -34 ±3 mm, pu, f; and -77 ±6 mm, pu, h were observed with the pH 9.4 buffer at 25 ma for 1.87 hr. Under these conditions, the aqueous layer from the first hydrazinolysis had ninhydrin positive bands at -54 ±4 mm, pu, h and -70 ±3 mm, pu, f. Serine and DAPA had bands at +13 ±8 mm and +7 ±5 mm, respectively.

The UV spectrum of this red gum had a λ_{max}^{HCl} at 265 mμ with an $E_{1cm}^{1\%}$ of ca. 36 and strong end absorption. There was no discreet λ_{max}^{NaOH}; there was strong end absorption with $E_{1cm}^{1\%}$ of ca. 24, 33, 43, and 53 at 300, 290, 280 and 270 mμ.

DNP-V_{maj}

A sample of 0.080 g of V_{maj} sulfate in five milliliters of water containing one gram of sodium bicarbonate was mixed with a solution of 0.5 g DNFB in ten milliliters of absolute ethanol. This solution was allowed to stand for two hours with occasional swirling. A yellow precipitate formed; the mixture was evaporated to about five milliliters; this mixture was acidified with 6 N hydrochloric acid and filtered. The DNP-V_{maj} obtained weighed 0.087 g; it was a bright yellow amorphous solid. The UV spectrum of this material had a $\lambda_{max}^{1\% \text{ DMF}}$ at 350 mμ ($E_{1cm}^{1\%}$, 235). There was strong end absorption at 290 mμ with an inflection at 265 mμ.

Characterization of V_{min}

Spectral Properties of V_{min}

A sample of V_{min} sulfate (27.2 mg, 0.034 mmoles) that had been dried for 72 hr at 80° under high vacuum was dissolved in water. The solution was diluted to 100 ml. Ten milliliter aliquots from this solution were diluted to 100 ml with 1 N hydrochloric acid, with water, and

with 1 N sodium hydroxide. The UV spectra of these solutions had $\lambda_{max}^{H_2O}$ at 269.5 mμ (ε, 22,300), λ_{max}^{HCl} at 269.4 mμ (ε, 27,400), and λ_{max}^{NaOH} at 290 mμ (ε, 16,200) with an assumed molecular weight of 800. The NMR spectrum of a 24 per cent solution of V_{min} sulfate (dried to constant weight) in D_2O was taken with DSS as internal standard. Absorptions centered around τ 2.2, 5.5, 6.4, 7.1 and 8.2 were observed. The absorptions at τ 6.4, 7 1, and 8.2 were poorly resolved multiplets; the absorption at τ 2.2 was a singlet. The IR spectrum of V_{min} sulfate was taken as a nujol mull; there was no major difference between this spectrum and that of V_{maj} sulfate.

Amino Acid Analysis of V_{min}

A sample of V_{min} sulfate was submitted for amino acid analysis; this was carried out as done previously with V_{maj}. The results, given as μmole/mg, were: serine 1.30, β-lysine 1.85, DAPA 1.01, viomycidine 0.893, and ammonia 1.95 with small peaks corresponding to cysteic acid, glycine, and alanine.

Complete Hydrolysis of V_{min}

A solution of 0.104 g of V_{min} sulfate was heated under reflux on the steam bath for 14 hr. The product, a dark red solution, was evaporated *in vacuo* to dryness; ten milliliters of water in two portions were added, and the solution was evaporated to dryness each time. The sample was then dried under high vacuum and dissolved in one milliliter of water. This solution was analysed by electrophoresis and TLC. Mobilities found for this hydrolysate and for standard solutions are shown in Table 6. The hydrolysate and a urea solution were chromatographed on the same CB plate in CB and the plate was then sprayed with pDMAB reagent. A bright yellow spot appeared immediately for urea at R_F 0.69. After the plate

Table 6. Electrophoresis Mobilities of the Acid Hydrolysate of V_{min}[a]

Hydrolysate	β-lysine	peptide III	viomycidine	DAPA	serine
-43 ±5 pu,h	-45 ±5 pu,m				
-21 ±5 br,f		-22 ±4 br,m			
-12 ±3 pu,f			-15 ±4 pu,f		
+12 ±8 pu,h				+18 ±8 pu,h	
+32 ±6 pu,h					+39 ±18 pu,h

[a]Conditions: pH 9.4, 15 ma for 2.5 hr, spray ninhydrin

was dry, a faint orange-yellow spot at R_F 0.64 appeared in the hydrolysate chromatogram. Paper chromatography of the hydrolysate, peptide III, and viomycidine was carried out in BAW. Very faint orange-red spots appeared at R_F values of 0.11 and 0.33. When the strip was sprayed with Weber reagent, viomycidine had an R_F value of 0.34 and peptide III had an R_F value of 0.12. Peptide III is one of the other guanidino compounds occurring in small quantities in the acid hydrolysate of viomycin.

Hydrazinolysis of V_{min}

A sample of 0.043 g of V_{min} sulfate was subjected to hydrazinolysis exactly as the sample of V_{maj} was treated previously. It was analysed at the same time as the V_{maj} hydrazinolysate; no difference was noted in the TLC or electrophoresis patterns of the hydrazinolysate of V_{maj} and V_{min}.

DNP-V_{min}

A sample of 0.061 g of V_{min} sulfate in five milliliters of water

containing one gram of sodium bicarbonate was mixed with 0.5 g of DNFB in ten milliliters of absolute ethanol. The ethanol was evaporated *in vacuo* after two hours; a yellow precipitate had formed during this time. The mixture was acidified with 6 N hydrochloric acid and filtered. The DNP-V_{min} obtained weighed 0.061 g; it was a bright yellow amorphous solid. The UV spectrum of DNP-V_{min} had a $\lambda_{max}^{1\%\ DMF}$ at 350 mμ ($E_{1cm}^{1\%}$, 238) with a shoulder on the end absorption at *ca.* 263 mμ.

Attempted Preparation of Desureaviomycin

Six Hour Hydrolysis

Following the procedure of Kellogg (5), a sample of 0.95 g (1.2 mmoles) of V_{maj} sulfate in 50 ml of 0.1 N hydrochloric acid was heated on the steam bath for six hours. The hydrolysate, a bright yellow solution, was neutralized to pH 7 with 50 ml of IR-45(OH^-). The mixture was filtered; the filtrate was evaporated to *ca.* 15 ml *in vacuo* at 70°C. A light yellow solid was obtained after lyopholization that weighed 0.837 g after it was dried overnight under high vacuum. This material was triturated with 20 ml of hot absolute ethanol; the mixture was filtered and the residue was washed with ten milliliters of hot ethanol. The residue weighed 0.828 g after it was dried. The filtrate was evaporated to dryness to give 0.004 g of an oil. Analysis by TLC showed that this oil contained at most a faint trace of urea; the residue gave two ninhydrin positive spots at R_F 0.0 and and R_F corresponding to pure V_{maj} on a CB plate in CB solvent. This mixture was chromatographed on a column filled to a height of 98 cm with 788 ml of Sephadex G-15. The column was eluted with 0.01 N formic acid; fractions of 9.5 ml in three minutes were taken.

Fractions 23 to 34 contained ninhydrin positive material. There was 0.239 g of R_F 0.0 material in fractions 23 to 26; the remainder of the material (0.616 g) gave two spots, R_F 0.0 and R_F 0.27, when analysed using CB plates and CB solvent. Pure viomycin gave one spot at R_F 0.28 on the same plate. The mobilities observed for this material upon electrophoresis at pH 9.4, five milliamperes for three hours were: R_F 0.0 material: -56 2 mm; mixture: -56 ± 2 mm and -82 ± 5 mm; pure V_{maj}: -82 ± 7 mm. The UV spectrum of fraction 25 had a $\lambda_{max}^{H_2O}$ at 279 mμ ($E_{1cm}^{1\%}$, 110) and the UV spectrum of fraction 29 had a $\lambda_{max}^{H_2O}$ at 279 mμ ($E_{1cm}^{1\%}$, 173).

Fractions 23-27 were combined and 0.13 g of this material was rechromatographed over the 550 cm column of Sephadex G-15 used for the purification of commercial viomycin. The column was eluted with 0.01 N formic acid; fractions of 19 ml in 5.1 min were taken. Fractions 19 to 26 contained ninhydrin positive material. Two distinct peaks were observed when a graph of fraction number vs fraction weight was made. The material in the first peak, fractions 19 to 22, weighed 0.087 g; it had one major component with a mobility of -61 ± 23 mm (pH 9.4, 15 ma for 2.5 hr). It had a broad unsymmetrical $\lambda_{max}^{H_2O}$ at 284 mμ ($E_{1cm}^{1\%}$, 150), and λ_{max}^{NaOH} at 285 mμ ($E_{1cm}^{1\%}$, 123) with a shoulder at 345 mμ. The material in the second peak, fractions 24 and 25, weighed 0.061 g; it had two major components with mobilities of -67 ± 5 mm and -78 ± 3 mm under the conditions given above. It had a $\lambda_{max}^{H_2O}$ at 276 mμ ($E_{1cm}^{1\%}$, 177) and a λ_{max}^{NaOH} at 285 mμ ($E_{1cm}^{1\%}$, 138) with no absorption at 345 mμ.

Eighteen Hour Hydrolysis

A sample of 1.438 g of V_{maj} sulfate in 50 ml of 0.1 N hydrochloric acid was heated for 18 hr at 90-100°. The light yellow solution was evap-

orated *in vacuo* to *ca.* 15 ml; the *p*H of this solution was brought to *p*H 6 with 75 ml of IR-45 (OH^-). This solution was evaporated to *ca.* 15 ml and then passed over a column of 104 ml of IR-45 ($SO_4^=$). The column was eluted with 400 ml of water. The effluent was evaporated *in vacuo* to *ca.* 40 ml. This solution gave a light yellow solid after lyopholization. The solid was extracted with absolute ethanol in a Soxhlet apparatus for ten hours. The ethanol solution gave 0.024 g of an oil when it was evaporated to dryness *in vacuo* at 70°. The dried residue from the extraction weighed 1.265 g. The oil gave a very faint orange-yellow *p*DMAB positive spot at an R_F of 0.75 on a CB plate in CB solvent; urea gave a bright yellow spot at an R_F of 0.75 under the same conditions. The residue gave one ninhydrin positive spot at R_F 0.0 in the same TLC system. It had two bands with electrophoresis mobilities corresponding to those of the material from the six hour hydrolysis. There was no trace of serine or β-lysine. The UV spectrum of this material had a very broad $\lambda_{max}^{H_2O}$ at 285-295 mμ ($E_{1cm}^{1\%}$, 135), a broad λ_{max}^{HCl} at 95 mμ ($E_{1cm}^{1\%}$, 138), and λ_{max}^{NaOH} at 284 mμ ($E_{1cm}^{1\%}$, 74) and 349 mμ ($E_{1cm}^{1\%}$, 107). A sample of 1.18 g of this material was chromatographed on the 550 cm Sephadex G-15 column used previously. The column was eluted with 0.01 *N* formic acid; 15 ml fractions were collected. Fractions 27 to 38 were lyopholized and weighed. Two peaks were observed in the chromatogram. The material in the first peak, fractions 27 to 34, weighed 0.815 g. Fraction 28 had a λ_{max}^{HCl} at 294 mμ ($E_{1cm}^{1\%}$, 177) and λ_{max}^{NaOH} at 288 mμ ($E_{1cm}^{1\%}$, 86) and 350 mμ ($E_{1cm}^{1\%}$, 150). The material in the second peak, fractions 35 to 38, weighed 0.108 g. Fraction 38 had a λ_{max}^{HCl} at 267 mμ ($E_{1cm}^{1\%}$, 93) and a λ_{max}^{NaOH} at 283 mμ ($E_{1cm}^{1\%}$, 86); there was no λ_{max}^{NaOH} at 350 mμ.

Comparison of the Product with and without an Ion Exchange Workup

A sample of 0.5 g of V_{maj} sulfate in 25 ml of 0.1 N hydrochloric acid was heated at 90-100°C for eight hours. One-half of this solution was lyopholized to an oil; this oil was dissolved in five milliliters of water and the solution was lyopholized to give a light yellow powder. The UV spectrum of this material was taken in water, 1 N hydrochloric acid and 1 N sodium hydroxide; it had a very broad $\lambda_{max}^{H_2O}$ at 296 mμ ($E_{1cm}^{1\%}$, 183); a broad λ_{max}^{HCl} at 297 mμ ($E_{1cm}^{1\%}$, 185), and λ_{max}^{NaOH} at 286 mμ ($E_{1cm}^{1\%}$, 77) and 350 mμ ($E_{1cm}^{1\%}$, 154).

The pH of the other half of the hydrolysate was brought to pH 5.5 with IR-45(OH^-); the mixture was filtered and the filtrate was evaporated *in vacuo* to *ca.* 15 ml. This solution was applied to a column of 100 ml of IR-45 ($SO_4^=$); the column was eluted with 250 ml of distilled water. The total effluent was evaporated *in vacuo* to *ca.* 15 ml; this solution was lyopholized to give a creamy-white powder. The UV spectrum of this material was taken in water, 1 N hydrochloric acid, and 1 N sodium hydroxide; it had a $\lambda_{max}^{H_2O}$ at 276 mμ ($E_{1cm}^{1\%}$, 138) with a shoulder at 295 mμ, a λ_{max}^{HCl} at 275 mμ ($E_{1cm}^{1\%}$, 161) with a shoulder at 296 mμ and λ_{max}^{NaOH} at 283 mμ ($E_{1cm}^{1\%}$, 110) and 348 mμ ($E_{1cm}^{1\%}$, 105).

Graphs of $E_{1cm}^{1\%}$ *vs* wavelength are shown in Figure 4 for the UV spectra in acid and in base of these two hydrolysate samples.

Hydrolysis of Viomycin With 6 N Hydrochloric Acid at Room Temperature

Release of Carbon Dioxide

Apparatus. The apparatus for the hydrolysis and measurement of the

carbon dioxide released consisted of a round bottom flask with a standard taper joint connected by glass tubing to a manometer and to a 500 ml gas measuring buret that could be filled with dibutyl phthalate from a reservoir. As carbon dioxide was released; the dibutyl phthalate level was changed to adjust the inner pressure to atmospheric pressure. The volume of gas released was read directly from the buret.

Model Compound: n-Butylcarbamate. A sample of 2.029 g (17.4 m moles) of n-butylcarbamate in 50 ml of 6 N hydrochloric acid was placed in the flask; after 24, 28, 96 and 119 hrs, 15, 25, 40 and 50 ml of gas had been released (5.8, 9.6, 15.3, and 19.3 mmoles).

Model Compound: Urea. A sample of 1.286 g of urea in 50 ml of 6 N hydrochloric acid was placed in the flask; no carbon dioxide was released during 72 hrs.

Viomycin. A sample of commercial viomycin sulfate in 50 ml of 6 N hydrochloric acid was placed in the flask. No carbon dioxide was released from this solution in 384 hrs (16 days).

Ultraviolet and Oxidative Studies on the Room Temperature 6 N Hydrochloric Acid Hydrolysate

Standard Solutions. Standard solutions of sodium arsenite (NaH_2AsO_3), sodium periodate ($NaIO_4$) and iodine were made according to Dyer (33). A 20 per cent by weight solution of potassium iodide in a saturated aqueous sodium bicarbonate solution was made. A one per cent solution of soluble starch in a three per cent boric acid solution was made.

Model Compound: Serine. A sample of 0.1058 g of L-serine was dissolved in 100 ml of a pH 6 buffer (sodium acetate-acetic acid). A ten milliliter aliquot from this solution was diluted to 100 ml with the

pH 6 buffer. Five milliliter aliquots of this solution were mixed with five milliliter aliquots of 0.00987 N sodium periodate solution; these solutions were allowed to react for various times. Ten milliliters of 0.01020 N sodium arsenite solution and ten milliliters of saturated sodium bicarbomate solution were added at the end of the reaction time. One milliliter of the starch solution and one milliliter of the potassium iodide solution were added; the solution was then titrated with a standard iodine solution. A blank solution was titrated for each sample. The number of moles of sodium periodate reacting per mole of serine was calculated. One mole of sodium periodate per mole of serine had reacted after two minutes; two moles of sodium periodate per mole of serine had reacted at 60 min.

Model Compound: DAPA. An 0.0011 M solution of DAPA·HCl was made up. Five milliliter aliquots of this solution were allowed to react with sodium periodate for varying times. At the end of the reaction time, the reaction was quenched with an excess of sodium arsenite solution (as with the serine samples), and the excess sodium arsenite was backtitrated with the iodine solution. One mole of sodium periodate had reacted after two minutes, and two moles had reacted after 120 min.

Viomycin. A solution of 1.5110 g (1.917 mmoles) of viomycin sulfate in 6 N hydrochloric acid was allowed to stand at room temperature for 18 days. At various times during this period, two and five milliliter aliquots were taken in order to determine the UV spectrum and the number of moles of sodium periodate that would be reduced. Each sample for sodium periodate oxidation was treated similarly to the samples of serine and DAPA mentioned previously. The equivalents of sodium perio-

date reduced with time for each sample were calculated; the equivalents of sodium periodate reduced after three hours reaction with each sample is shown in Table 7. The two milliliter samples for UV spectra were diluted to 100 ml with distilled water. Dilutions were made with water, pH 7 buffer, and 0.1 N sodium hydroxide solutions to give a concentration equivalent to 0.00362 g of the original viomycin per 100 ml. The UV spectra of these solutions were taken and the λ_{max} and extinction coefficient for each solution are shown in Table 7.

Isolation of Peptides from the Room Temperature 6 N Hydrochloric Acid Hydrolysate of Viomycin

Isolation with Ion Exchange Resins

The yellow solution from the 16 day hydrolysis of viomycin at room temperature in 6 N hydrochloric acid that was carried out in order to determine the amount of carbon dioxide released* was evaporated to dryness in vacuo at room temperature to give a yellow glass. The glass was dissolved in 200 ml of water, and the pH of the solution was brought to pH 5.5 with IR-45 (OH^-). The mixture was filtered and the filtrate was evaporated to dryness in vacuo to give a yellow amorphous powder. Analysis by TLC proved that there was no viomycidine or DAPA present in the hydrolysate. A sample of 7.362 g of the hydrolysate in ten milliliters of water was applied to a column of 100 ml of IRA-400 (OH^-) in a 100 ml buret. The column was eluted with ca. 100 ml of freshly boiled distilled water. After this solution was evaporated in vacuo to 25 ml, it was applied to 90 ml of IRC-50 (H^+) in a 100 ml buret. The IRC-50 column was

*See this thesis, page 34.

Table 7. UV Spectra and Equivalents of Sodium Periodate Reduced for the 6 N Hydrochloric Acid Room Temperature Hydrolysate

Time (days)	Moles of Sodium Periodate Reduced per Mole of Viomycin (Three Hours Reaction)	UV Spectra - $E^{1\%}_{1\ cm}$ [a] pH 7		pH 12	
		268 mμ	297 mμ	275 mμ	348 mμ
0.0	0.77	260	32	166	0
0.062	----	220	39	155	0
0.17	----	188	50	147	11
0.25	0.92	178	65	141	19
0.33	----	142	65	135	33
0.50	----	114	82	120	39
1.0	1.15	80	106	130	79
2.0	1.34	69	105	112	83
4.0	1.09	50	69	90	51
7.0	1.67	35	41	67	28
10.5	1.82	30	30	55	19
15.0	2.23	22	14	41	10
18.0	2.38	22	14	36	8

[a]The λ_{max} at 268 mμ was shifted slightly (268 to 272 mμ) as the nearby λ_{max} at 297 mμ increased in intensity; the absorption at 268 mμ was estimated graphically.

eluted with ca. 175 ml of distilled water; this solution was evaporated to a small volume and passed over 50 ml of IR-45 ($SO_4^{=}$) to give the stoichiometric sulfate. The effluent from the IR-45 column was lyopholized to give 3.22 g of a light yellow fluffy material (the neutral fraction). Elution of the IRA-400 column with ca. 320 ml of 0.24 N hydrochloric acid followed by conversion of the anion of the effluent to sulfate with an IR-45 ($SO_4^{=}$) column gave a red solution which yielded, after lyopholization, 3.34 g of a brown glass (the acidic fraction). Elution of the IRC-50 column with ca. 200 ml of 0.24 N hydrochloric acid followed by conversion of the anion of the effluent to sulfate gave a red solution from which 1.60 g of a light brown solid (the basic fraction) was obtained by lyopholization. The result of analysis of these fractions by electrophoresis is in Table 8. When analysed by TLC, the neutral and basic fractions gave only one spot at an R_F value of 0.0; this spot was ninhydrin, UV, and Weber positive. These fractions are mixtures of the two components that do not correspond to any of the degradation products of viomycin found previously. The component of higher mobility at pH 9.4 will be called Peptide I; the component of lower mobility will be called Peptide II.

A sample of 0.10 g of the neutral fraction in 0.75 ml of water was applied to a column of 25 ml of Dowex 50 (H^+) in a 25 ml buret. The column was eluted with 20 ml of water, 80 ml of 1 N hydrochloric acid, and 30 ml of 3 N hydrochloric acid. The residue obtained from the aqueous eluate after evaporated weighed 0.053 g; it gave a heavy band corresponding to peptide I and a faint band corresponding to peptide II when it was analysed by electrophoresis. When this material was hydrolysed in 6 N hydrochloric acid overnight at 90°, it gave a mixture. This mixture gave

Table 8. Electrophoresis Mobilities of Fractions from the Room Temperature 6 N Acid Hydrolysate of Viomycin and of Standards.[a]

Acidic Fraction	Basic Fraction	Neutral Fraction	β-lysine	Peptide III	Viomycidine	DAPA	Serine
-75±8h			-74±8				
	-68±9h[b]	-63±7h[b]					
-49±5m							
-34±5m				-31±5[b]			
-16±7m[b]	-17±6h[b]	-12±6m[b]			-14±6[b]		
						+19±9	
							+40±13
+30±15H							
+70±8f							

[a]Conditions: ten milliamperes for four hours at pH 9.4; spray: ninhydrin.

[b]These bands were also Weber positive.

four spots when it was analysed by TLC (CB plate, CB solvent) with R_F values of 0.22 br, 0.38 pu, 0.55 pu, 0.69 pu. Peptide III, β-lysine, viomycidine, DAPA, and serine had R_F values of 0.22; 0.34; 0.38 and 0.54; 0.49; 0.70, respectively. The 3 N hydrochloric acid eluate was evaporated to give 0.018 g of a dark red glass. It gave a heavy band corresponding to peptide II and a faint band corresponding to peptide I when it was analysed by electrophoresis. This material gave a mixture when it was hydrolysed in 6 N hydrochloric acid overnight at 95°. This mixture was analysed by TLC at the same time as the mixture from the hydrolysis of peptide I. It had four spots at R_F values of 0.22, 0.37, 0.57, and 0.70.

Purification of Peptide I with Sephadex G-10.

A solution of 2.06 g of the mixture of peptide I and peptide II in about four milliliters of hot water was applied to a column 1.4 cm I.D. filled to the height of 132 cm with Sephadex G-10 which had been hydrated with 0.01 N formic acid. The column was eluted with 0.01 N formic acid; fractions of six milliliters in 15 min were collected. Fractions 17 to 24 were lyopholized, weighed, and analysed by electrophoresis. Fractions 17 and 18 contained 0.444 g of peptide I (-89 ± 14 mm at pH 9.4, 4.5 hr at five milliamperes); Fractions 19 to 24 contained mixtures of peptide I and peptide II (-8 ± 6 mm). There was a faint trace of a third component in fractions 19 and 20 (-118 ± 3 mm).

Isolation of Peptide I Using Gel Filtration

A sample of 6.80 g of commercial viomycin sulfate was allowed to stand at room temperature for 20 days in 35 ml of 6 N hydrochloric acid. The dark brown solution was then evaporated to dryness in vacuo at 35° to

give a brown glass. A sample of ca. 5.3 g of this material in water was applied to a column 3.5 cm I.D. filled to a height of 228 cm with Sephadex G-10. The column was eluted with 0.01 N formic acid. Fractions of 15 ml were collected; fractions 38 to 74 contained ninhydrin positive material. The first peak, fractions 38 to 42 contained 1.32 g of a mixture which gave three bands at -50 ± 5 mm, -10 ± 3 mm, and +45 ± 2 mm (pH 9.4, three hours at ten milliamperes); this mixture had one spot at an R_F value of 0.0 on a CB plate in CB solvent. These fractions were combined and re-chromatographed on the 550 cm Sephadex G-15 column which had previously been used to purify commercial viomycin. The column was eluted with 0.01 N formic acid. Fractions of 19 ml in 24 min were collected; fractions 22 to 38 contained ninhydrin positive material; fractions 22 to 24 contained 0.495 g of pure peptide I. These fractions were combined. The material had one band at -51 ± 3 mm; peptide I isolated earlier had one band at -52 ± 3 mm (pH 9.4, 15 ma for three hours). The remaining fractions were mixtures of peptide I and peptide II.

Characterization of Peptide I

Physical Tests

Peptide I sulfate was a white, fluffy solid when it was isolated by lyopholization. It decomposed when heated above 200°C. It was moderately soluble in water (only ca. 0.04 g dissolved when 0.080 g was added to 0.05 ml of water). It was insoluble in seven per cent aqueous sodium bicarbonate. A solution of peptide I in water was faintly red-brown at first. Upon standing for several months, the solution turned darker brown; TLC of such a sample had a heavy spot at R_F 0.0 and a faint spot

at R_F 0.50. Serine had R_F 0.50 on the same plate (BAcAAmW solvent).

The UV spectrum of a sample of peptide I sulfate that had been stored over phosphorous pentoxide for three weeks was taken in 1 N hydrochloric acid, water, and 1 N sodium hydroxide. It had a λ_{max}^{HCl} at 294 mμ ($E_{1cm}^{1\%}$, 246) a $\lambda_{max}^{H_2O}$ at 294 mμ ($E_{1cm}^{1\%}$, 233), and λ_{max}^{NaOH} at 291 mμ ($E_{1cm}^{1\%}$, 99), and 348 mμ ($E_{1cm}^{1\%}$, 158). The NMR spectrum of peptide I sulfate was taken as a six per cent solution in deuterium oxide. Absorptions at τ 1.9, 5.2, 5.6, 6.0, 7.0, and 8.2 had relative intensities (by planimeter) of 1:17:2:6:2:3. All peaks were broad and poorly resolved.

Complete Hydrolysis of Peptide I

A sample of 0.039 g of peptide I sulfate in one milliliter of 6 N hydrochloric acid was heated under reflux on the steam bath overnight. The dark brown solution was evaporated to dryness in vacuo to give a dark brown glass. The glass was dissolved in ten milliliters of water and evaporated to dryness again. A solution of this brown glass was analysed by TLC along with standard solutions; the results are in Table 9. No spot gave a positive test with pDMAB reagent.

Hydrazinolysis of Peptide I

A sample of 0.086 g of peptide I hydrobromide in two milliliters of anhydrous hydrazine in a sealed tube was heated for 18.5 hr at 115-120°. The tube was cooled and opened. The light yellow solution was evaporated to dryness over concentrated sulfuric acid in vacuo to give a tan glass. This glass dissolved completely in one milliliter of water; ten drops of pH 7 buffer and one milliliter of redistilled benzaldehyde were added. This mixture was allowed to stand overnight with occasional shaking. The mixture was then evaporated in vacuo to dryness at 70° to

Table 9. R_F Values of the Acid Hydrolysate of Peptide I.

Hydrolysate a	Hydrolysate b	Hydrolysate c	serine	DAPA	viomycidine	β-Lysine	Peptide III
0.58 rp	0.75[d]	0.45 rp	0.57 rp[a]	0.41 br[a]	0.45 p, 0.27 rp[a]	0.26 p[a]	0.16 br[a]
0.46 p	0.69 p	0.32 p	0.72 rp[b]	0.78 br[b]	0.76 p, 0.36 rp[b]	0.31 p[b]	0.19 br[b]
0.40 br	0.34 rp	0.28 br	0.42 rp[c]	0.25 br[c]	0.32 p, 0.20 rp[c]	0.15 p[c]	0.04 br[c]
0.28 rp	0.16 br	0.19 rp					
0.16 br		0.04 br					

[a]Conditions: CB plate, CB solvent, ninhydrin spray

[b]Conditions: CB plate, water as solvent, ninhydrin spray

[c]Conditions: CB plate, BAW solvent, ninhydrin spray.

[d]This spot was brown at the top and purple at the bottom.

give a brown gum which was only partially soluble in a mixture of water and ether. This mixture was filtered, and the aqueous layer was separated; it was extracted again with 50 ml of ether. The aqueous layer was then evaporated *in vacuo* to give 0.073 g of a tan glass that was redissolved in ten drops of water and analysed by TLC (BAAmW solvent). There was a red-purple ninhydrin positive spot at an R_F value of 0.60. Serine had an R_F value of 0.59, DAPA had an R_F value of 0.52, and viomycidine had an R_F value of 0.53 on the same plate. A second TLC plate was run in BAW; the material from the hydrazinolysis had an R_F value of 0.44; serine, DAPA, and viomycidine had R_F values of 0.44, 0.32, and 0.29 and 0.22 (two spots for viomycidine), respectively.

DNP-Peptide I

Preparation. A sample of 0.053 g of peptide I was added to 25 ml of water containing one gram of sodium bicarbonate. A fluffy white precipitate formed immediately. A solution of 0.150 g of DNFB in 25 ml of absolute ethanol was added to this, and the mixture was allowed to stand for four days with occasional swirling. The original white precipitate gradually changed to a bright yellow precipitate during this time. Concentrated hydrochloric acid was added until the mixture was acidic. The mixture was evaporated *in vacuo* at 50° to *ca.* 25 ml and filtered; the residue was washed with 6 *N* hydrochloric acid, water, and ethanol to give 0.070 g of a dull yellow amorphous solid. The DNP-peptide I was insoluble in water, ethanol, ethyl acetate, ether and chloroform. It was slightly soluble in boiling acetic acid and in hot ethylene glycol; it was very soluble in DMF and DMSO. Attempts to crystallize DNP-peptide I from various solvent mixtures failed. The material had an R_F value of 0.0 in all

solvent systems used (BAW; five per cent DMSO in methanol; 20 per cent DMSO in methanol; DMF:t-butyl alcohol::1:4; t-amyl alcohol:ethanol:concentrated ammonium hydroxide::80:7:20; BAAmW). The DNP-peptide I was routinely prepared using this procedure.

Hydrolysis of DNP-peptide I. A sample of 0.10 g of DNP-peptide I partially dissolved in seven milliliters of concentrated hydrochloric acid in a sealed tube was heated for 17 hr in an oil bath at 96°. The tube was cooled and opened; the dark red-brown solution was evaporated to dryness in vacuo at 30-40°. The gummy red-brown solid was triturated first with ethyl acetate, then with 1-butanol, and then with water. A small amount of insoluble black material remained. When these three extracts were evaporated to dryness, only the water and 1-butanol extracts contained material. These two extracts were analysed by TLC, paper chromatography, and electrophoresis.

Standards that were used in this analysis were serine, DAPA, viomycidine, peptide III, DNP serine, DNP peptide III, bis-DNP-DAPA, and β-DNP-DAPA. The bis-DNP-DAPA was prepared according to Kellogg's procedure (5); the β-DNP-DAPA was prepared using a procedure modified from that used by Sanger to prepare δ-DNP-ornithine (33). A sample of 1.9 g of DAPA·HCl was dissolved in 25 ml of boiling water. Copper carbonate was added until no more dissolved; a slight excess was added, and the mixture was heated for five minutes more. The mixture was filtered through Celite to give a dark blue solution; this solution was evaporated in vacuo to ca. 20 ml. A quantity of 1.1 g of sodium bicarbonate was added to the solution. A small amount of a light blue precipitate formed immediately. The cloudy supernatent solution was light green.

After standing for two hours, the precipitate was separated by centrifugation and the solution was evaporated to ca. 15 ml. A yellow precipitate formed. This was separated by filtration. The filtrate was acidified and allowed to stand in the refrigerator. A heavy precipitate formed that was determined to be DAPA by TLC; this precipitate was separated by filtration and the filtrate was evaporated to dryness. Analysis by TLC showed that there was one ninhydrin positive DNP compound present in the residue. This was purified by preparative TLC using 0.025 mm thick silica gel HF_{254} plates in the solvent system benzyl alcohol:ethanol:concentrated ammonium hydroxide::80:40:60. The yellow ninhydrin positive streak on the TLC plates was scraped off, and the silica gel was extracted with DMF. The DMF solution was evaporated to dryness in vacuo to give 0.056 g of a dark red-brown solid. This material was analysed by paper chromatography; it had an R_F value of 0.63 in n-butyl alcohol:acetic acid:water::2:1:1 and 0.66 in n-butyl alcohol:t-butyl alcohol:pyridine:acetic acid:water:: 15:4.5:10:3:12. The literature values are 0.76 and 0.53 (24).

The data for analysis of the aqueous extract of the hydrolysate of DNP-peptide I and standards is shown in Table 10. The TLC and electrophoresis patterns for the material extracted by 1-butanol and water were identical except for the intensities of the spots.

Hydrazinolysis of DNP peptide I. A sample of 0.153 g of DNP-peptide I was dried overnight at 80° under high vacuum and dissolved in one milliliter of anhydrous hydrazine to give a deep red solution. This solution was sealed in a thick walled ampule and heated for 16.7 hr at 90-105°. The tube was opened and the deep red solution was evaporated to dryness in vacuo over concentrated sulfuric acid to give a dark red-

Table 10. Electrophoresis Mobilities and TLC R_F Values for the Hydrolysates of DNP-Peptide I and Standard Solutions

[a]Hydrolysate	Serine	DAPA	Viomycidine	DNP-Serine	β-DNP DAPA	DNP Peptide I
[b]0.0 y, pu; 0.03, pu; 0.19 y, br; 0.20 y	0.03 pu	0.0 br	0.02 pu	0.19 y		0.0 y
[c]0.21 br; 0.38 pu; 0.47 y, pu; 0.64 y	0.48 pu	0.40 br	0.37 pu			
[d]0.0 pu; 0.16 br; 0.49 y, br, 0.65, y					0.49 y, pu	
[e]-17 ± 8 mm, pu; +45 ± 8 mm, pu; +62 ± 8 mm, y, pu	+44 ± 8 mm, pu	+21 ± 8 mm, pu	-24 ± 12 mm, pu	+64 ± 6 mm, y		
[f]0.23 pu; 0.36 pu; 0.65 y, br	0.36 pu		0.25 pu		0.64 y, br	

[a]The spots that were yellow before spraying are designated y; the usual abbreviations are used for ninhydrin positive spots.

[b]TLC : t-amyl alcohol:ethanol:concentrated ammonium hydroxide::80:7:20.

[c]TLC : BAAmW solvent

[d]TLC : benzyl alcohol:ethanol:concentrated ammonium hydroxide::80:60:40.

[e]Electrophoresis:pH 9.4, five milliamperes for 16 hr.

[d]Paper chromatography:n-butyl alcohol:acetic acid: water::2:1:1.

brown gum that was then dissolved in one milliliter of water. One milliliter of pH 7 buffer and one milliliter of redistilled benzaldehyde were added and the mixture was allowed to stand overnight with occasional stirring. A black precipitate formed in the mixture. The mixture was filtered, and the filtrate was extracted with two 10-ml portions of ether. The aqueous layer was evaporated to ca. 0.5 ml. The results of TLC and electrophoresis analyses are given in Table 11.

Hydrolysis of Viomycin with Dowex-1 Anion Exchange Resin

Hydrolysis

A sample of 5.08 g (6.35 mmoles) of viomycin sulfate was dissolved in 50 ml of distilled water. A quantity of 20 ml of Dowex-1X8 (OH^-) strongly basic anion exchange resin (reagent grade, 100-200 mesh) was added, and the mixture was heated under reflux at the boiling point. After one day, the mixture was analysed by TLC (BAW as solvent). A purple spot at an R_F value of 0.58 and a purple streak from the origin to R_F 0.35 was observed. On the same plate, serine, β-lysine, DAPA, and viomycidine had R_F values of 0.58, 0.19, 0.34 and 0.58 and 0.26. There was no brown spot in the hydrolysate chromatogram that appeared immediately after spraying such as DAPA gave. The streak from R_F 0 to R_F 0.3 was Weber positive. No changes appeared in the TLC pattern of the hydrolysate on the second through the sixth days except that the intensity of spots with R_F values corresponding to β-lysine and serine became greater. The UV absorption of the solution at 267 mμ decreased with time. After one hour, the $E^{1\%}_{1cm}$ was 236; after 24 hr. it had decreased to 107, and after 96 hr it had decreased to 38. The hydrolysis was ended after six

Table 11. Electrophoresis Mobilities and TLC R_F Values for the Analysis of the DNP-Peptide I Hydrazinolysate.

Hydrazinolysate of DNP-Peptide I[a]	Serine	Hydrazinolysate of V_{maj}[a]
[b]0.82 y-br, 0.71 pu	0.69 pu	0.85 y-br, 0.64 pu
[c]0.80 y-br, 0.64 pu	0.64 pu	0.81 y-br, 0.67 pu
[d]0.70 y-br, f; 0.56 pu, h	0.57 pu	0.70 y-br, 0.56 pu
[e]-70 ± 2 mm, pu, f; -55 ± 3 mm, pu, m; -8 ± 2 mm, y-br, f; +10 ± 4 mm, pu, m	+ 13 ± 8 mm, pu	-69 ± 2 mm, pu, f; -54 ± 4 mm, pu, m; -5 ± 6 mm, y-br, f

[a]The spot marked y-br was yellow-brown before spraying.

[b]TLC : BAcAAmW solvent.

[c]TLC : BAW solvent.

[d]TLC : CB plate, CB solvent.

[e]Electrophoresis: pH 9.4, 25 ma for 1.87 hr.

days. The mixture was poured into a 100 ml buret; the resin was eluted with 100 ml of distilled water. This solution was evaporated to dryness at ca. 50° in vacuo to give 3.47 g of a tan solid. The resin was then eluted with ca. 100 ml of 1 N hydrochloric acid; this solution was evaporated to dryness at ca. 50° in vacuo to give 0.070 g of a dark red oil. The material eluted from the Dowex-1 resin with water was fractionated by chromatography on a 50 ml column of IRA-400 (OH^-) resin connected directly to the top of a 50 ml IRC-50 (H^+) column. The columns were eluted with 100 ml of distilled water. The effluent was evaporated to dryness in vacuo to give ca. 0.05 g of a red-brown oil. The columns were separated, and the IRA-400 column was eluted with 100 ml of 1 N hydrochloric acid; the effluent was evaporated to a small volume. The pH of the solution was brought to pH 5 with IR-45 (OH^-), and it was chromatographed on a 30 ml column of IR-45 (Cl^-). The effluent from this column was evaporated in vacuo to dryness to give 0.851 g of a dark red solid, the acidic fraction. The IRC-50 column was eluted with 100 ml of 6 N hydrochloric acid. The effluent was evaporated to pH 5 with IR-45 (OH^-). The solution was then chromatographed on a 30 ml column of IR-45 (Cl^-), the effluent from this column was evaporated to dryness in vacuo to give 2.32 g of a purple solid, the basic fraction. Analysis of these fractions by TLC was carried out using a silica gel H plate developed in water. The acidic fraction had a heavy purple spot at an R_F value of 0.97 and a streak from R_F 0.0 to R_F 0.26 which was ninhydrin and Weber positive. The basic fraction had only a ninhydrin and Weber positive streak from R_F 0.0 to R_F 0.43. On the same plate, serine, β-lysine, and viomycidine had R_F values of 0.97, 0.0, and 0.90 respectively.

Further Purification of the Basic Fraction

The basic fraction was then rechromatographed on a 100 ml IRC-50 (H^+) column. The aqueous effluent from this column was evaporated to dryness to give 1.23 g of a brown solid, which still gave several spots when it was analysed by TLC. This material was then rechromatographed on an 87 ml column of IRA-400 (OH^-). The aqueous effluent from this column was evaporated to dryness to give 0.76 g of a tan solid which gave one ninhydrin and Weber positive spot at R_F 0.0 (CB plate, CB solvent). This material had one major band at -69 ± 5 mm when it was analysed by electrophoresis at pH 9.9 at ten milliamperes for four hours. A sample of 0.085 g of this material in five milliliters of 6 N hydrochloric acid was heated on a steam bath for six hours. The solution had ninhydrin positive spots at R_F values of 0.65 pu, h; 0.51 pu,h; 0.35 h; 0.16 br, h. Serine, DAPA, β-lysine, and viomycidine had R_F values of 0.65, 0.40, 0.31, and 0.51 and 0.34, respectively, on the same plate (CB plate, CB solvent). Electrophoresis of the hydrolysate and standard solutions was carried out at pH 9.4 at ten milliamperes for four hours. The hydrolysate had bands at 26 ± 10 mm, +15 ± 5 mm, and +40 ± 5 mm; β-lysine, peptide III, viomycidine, DAPA, and serine had bands at -70 ± 5 mm, -30 ± 5 mm, -13 ± 5 mm, +18 ± 7 mm, and +39 ± 10 mm respectively.

An attempt was made to purify this material further using a Darco-Celite column (120 ml in a 3.8 cm I.D. x 212 cm high column). The material was not eluted with water, two per cent acetone in water, and 0.2 N hydrochloric acid; a small amount of serine was eluted by water. A significant amount of material (ca. 30 per cent recovery) was eluted by ten per cent acetone in 0.1 N hydrochloric acid. This material was shown to

have several components by TLC and electrophoresis.

Purification of the Basic Fraction by Gel Filtration.

The basic fraction (0.817 g) which had been separated by ion exchange chromatography from the Dowex-1 (OH^-) hydrolysate of 5.8 g of viomycin hydrochloride was purified further on a 1450 ml Sephadex G-10 column. The column was eluted with 0.01 N formic acid; fractions of 15 ml in ten minutes were collected. Fractions 33 to 54 contained ninhydrin positive material. The main component (ca. 390 mg in fractions 44 to 49) had an electrophoresis mobility of -66 ± 3 mm; authentic peptide I had a mobility of -65 ± 5 mm at the same time (pH 9.4, 25 ma for three hours).

Peptide III

Purification of Peptide III

Besides viomycidine, another guanidino compound was reported to occur among the complete acid hydrolysis products of viomycin (5). A quantity of this material*, referred to earlier as "the lower R_F guanido compound", was characterized and used as a reference compound when more of the material was purified. The material will be referred to as "Peptide III" in this thesis.

Floyd's method for purification of peptide III (gradient elution of the gross hydrolysate of viomycin from a Dowex 50 (H^+) column with hydrochloric acid solutions) was the best method for separation of peptide I from the other hydrolysis products (12). In a typical preparation, ca. 18 g of commercial viomycin sulfate in 50 ml of 6 N hydrochloric

*This material was obtained from Thomas Bickley, September, 1964. It had been purified by ion-exchange chromatography and by chromatography on a Darco-Celite column.

acid was heated for seven hours under reflux on a steam bath. The sample was then evaporated *in vacuo* at 70° to give a dark brown oil which was redissolved in a small volume of 1 N hydrochloric acid. A column of 2440 ml of Dowex 50 (H^+) in a 7.5 cm I.D. column was prepared. The sample was applied to the top of the column, and the column was eluted with one liter of 1 N hydrochloric acid. Fractions of 17.5 ml were collected. The gradient elution apparatus was then set up. A reservoir containing two liters of 1 N hydrochloric acid was attached by plastic tubing to the top of the column, which already had about one liter of 1 N hydrochloric acid above the resin. The two liter reservoir was stirred magnetically. The more concentrated 5 N hydrochloric acid was added to this reservoir from a second reservoir at the same rate that the column was eluted. A total of 630 fractions were collected; these fractions were pooled in groups of ten fractions and evaporated to dryness *in vacuo* at 70°. The excess hydrochloric acid was removed by the addition of redistilled *t*-butyl alcohol to the pooled fractions, followed by evaporation *in vacuo* of the mixture of *t*-butyl chloride and *t*-butyl alcohol which was formed. The fractions were analysed by TLC. Fractions 521-610 contained material with an intense brown spot at R_F 0.12; the reference solution of peptide III had an intense brown spot at R_F 0.13 (CB plate, BAW solvent). A faint purple spot at R_F 0.3 was present in most of the fractions. The fractions containing peptide III were further purified by chromatography on a Darco-Celite column (864 ml in a 5 cm I.D. column). The column was eluted with deionized water. A total of 1.45 g of impure peptide III was recovered from the column.

A sample of 1.35 g of this impure peptide III was then chroma-

tographed on a 1450 ml Sephadex G-10 column. The column was eluted with 0.01 N formic acid; fractions of 15 ml in nine minutes were collected. Fractions containing ninhydrin positive material were lyopholized and weighed. A total of ca. 0.5 g of peptide III, which was homogenous by TLC, was obtained from this separation.

Preparation and Crystallization of Peptide III Hydrobromide

A sample of ca. 1.0 g of crude peptide III hydrochloride was partially neutralized to pH 6 batchwise with ca. 40 ml of IR-45 (OH^-). This mixture was filtered and the filtrate was evaporated to ca. 15 ml in vacuo. This solution was applied to a 150 ml column of IR-45 (Br^-); the column was eluted with ca. 350 ml of water to give a red solution. The solution was decolorized by heating it with ca. one gram of Darco. The mixture was then filtered, and the filtrate was evaporated to ca. 40 ml. This solution was heated to boiling, and ethanol was added until the solution became slightly cloudy; after boiling for a few minutes, the solution became clear again and n-butyl alcohol was added to it until the solution became cloudy again. About 50 ml of ethanol and 15 ml of n-butyl alcohol were used. The solution was allowed to stand at 0° for four days; crystallization occurred during this time. After further crystallization of the mother liquor, enough material was obtained so that, after two recrystallizations, sufficient material was obtained for elemental analysis. The crystals were white needles, mp 200-205°C dec. A sample was dried to constant weight in vacuo at 25° and analysed for C, H, N, and Br.

Anal.: $C_9H_{15}N_5O_3 \cdot 2\ HBr$

(405.1)

Calc'd.: C, 26.79; H, 4.24; N, 17.37; Br, 39.65

Found: C, 26.46; H, 4.54; N, 17.35; Br, 39.72

(lost 12.53 per cent on drying)

A total of ca 0.3 g of crystalline peptide III•2 HBr was obtained from a second preparation of peptide III hydrobromide, starting with ca. 1.36 g of peptide III hydrochloride. This material was recrystallized two times from acetone-water; a crystal in the material from the second recrystallization was suitable for an X-ray structure determination.* A sample of this material was air dried and analysed for C, H, and N. Another sample from the same vial was dried to constant weight in vacuo at 25° and analysed for C, H, N, and Br.

Anal. Found: C, 24.11; H, 5.51; N, 16.03

(air dired)

Found: C, 24.80; H, 4.67; N, 17.84; Br, 37.75

(lost 8.03 per cent on drying)

The UV spectrum of crystalline peptide III•2 HBr was taken in water at a concentration of 9.0 mg per 100 ml (0.090 per cent). There was no absorption except for strong end absorption beginning at ca. 210 mμ.

The NMR spectrum of a sample of peptide III hydrochloride was taken as a 16 per cent solution in D_2O with DSS as internal standard. This sample of peptide III hydrochloride was homogenous by TLC paper chromatography, and electrophoresis; it was an amorphous white solid. It had been purified by ion exchange chromatography and chromatography on a Darco-Celite column. The NMR spectrum had broad absorptions centered a-

*This X-ray structure determination is being carried out by F. L. Suddath.

round τ 7.40, 7.05, 6.42, 6.30, 6.13, 6.03, ca. 5.5, and 4.63. The NMR spectrum of crystalline peptide III•2 HBr was taken as a seven per cent solution in D_2O with DSS as internal standard at room temperature and at 80°. By taking the spectrum at 80°, the peak due to HOD was shifted 26 cps upfield from its position at room temperature, and absorptions that had been hidden behind it were revealed. In addition, all other peaks in the spectrum were shifted 5.5 cps upfield in the spectrum run at 80°. A composite spectrum of the non-exchangeable protons is shown in Figure 5. The absorptions in the 80° spectrum are corrected for the temperature shift. Spinning side bands were identified by changing the spinning speed. They were deleted from the spectrum. The spectrum was expanded at room temperature and integrated using a planimeter. The absorptions at τ 4.5 (doublet), 5.2 (complex multiplet) and 6.0 (complex multiplet) had relative areas of 1:3.9:5.7.

Typical electrophoresis mobilities for peptide III•2 HBr were -91 ± 9 mm, br (pH 4, five milliamperes for three hours) and -30 ± 5 mm, br (pH 9.4, ten milliamperes for four hours).

Hydrolysis of Peptide III

A sample of 0.038 g of peptide III hydrochloride that was homogenous by TLC was dissolved in two milliliters of 6 N hydrochloric acid; this solution was heated for six hours on a steam bath under reflux. The hydrolysate was analysed by TLC using silica gel H plates in several solvent systems; the results are in Table 12.

A second sample of 0.053 g of peptide III in 1.0 ml of 6 N hydrochloric acid was heated for 16 hr under reflux on a steam bath. Analysis of this hydrolysate by TLC gave essentially the same patterns as the anal-

Table 12. TLC Analysis of the Acid Hydrolysate of Peptide III.

Hydrolysate	Peptide III	DAPA	Viomycidine	Serine
0.08 br, 0.18 pu, 0.28[a] (a streak)	0.08 br[a]	0.28 br[a]	0.18 rp, 0.28 p[a]	0.39 p[a]
0.24 br, 0.35 to 0.70 pu, 0.88 br[b]	0.24 br[b]	0.88 br[b]	0.55 rp, 0.74 p[b]	0.92 p[b]
0.29 to 0.73, or-r streak[c]	0.29[c]		0.50 or-r, 0.63 or-r[c]	

[a]TLC : BAW solvent, ninhydrin spray. The spot in the hydrolysate at R_F 0.28 was brown immediately after spraying; it turned to a red brown color later.

[b]TLC : H_2O solvent, ninhydrin spray.

[c]TLC : H_2O solvent, Weber spray.

ysis of the six hour hydrolysate except that the intensity of the spot corresponding to peptide III was less and the intensities of the DAPA and viomycidine spots were greater.

DNP-Peptide III

In a typical preparation of DNP peptide III, 0.517 g of peptide III in 15 ml of distilled water containing one gram of sodium bicarbonate was mixed with a solution of one gram of DNFB in 15 ml of absolute ethanol. A yellow precipitate began to form immediately; the mixture was allowed to stand with occasional shaking for two days. It was then acidified and evaporated to ca. one-half the original volume in vacuo. The mixture was then filtered; the bright yellow amorphous solid was washed copiously with

cold 1 N hydrochloric acid, cold water, and with five milliliters of cold ethanol. After it was dried the material weighed 0.693 g. Since it gave three spots at R_F 0.67 y, h; 0.81 y, f; and 0.89 y, f (TLC, BAW solvent), the material was further purified using a column of 200 g of 25 per cent celite in silicic acid packed as a dry solid to a height of 52 cm in a 3.4 cm I.D. x 60 cm high column. A sample of 0.691 g of DNP-peptide III in one milliliter of DMF was washed on the column with two 2-ml portions of DMF. The column was then eluted with 700 ml of redistilled acetonitrile, 70 ml of 1:1 DMF-acetonitrile, and 400 ml of DMF. Twelve 100-ml fractions were collected; the DNP-peptide III was eluted in fractions 7-11; these fractions were pooled and evaporated to dryness in vacuo at 70°. The red gummy solid weighed ca. 0.5 g; it was dissolved in one milliliter of DMF. This solution was added drop by drop to 500 ml of ether as it was stirred magnetically. The bright yellow amorphous precipitate was filtered, washed with ether, and dried to give 0.40 g of DNP-peptide III (mp 236° dec). This material had only one spot in several TLC systems (BAW, R_F 0.66; BAAmW, R_F 0.70; t-amyl alcohol:ethanol:concentrated ammonium hydroxide::80:7:20, R_F 0.08). The DNP-peptide III was insoluble in hot acetic acid, and it was very soluble in DMF and DMSO. Efforts to crystallize it from several different solvent systems failed.

Hydrolysis of DNP-Peptide III

A sample of 0.059 g of DNP-Peptide III in five milliliters of concentrated hydrochloric acid in a sealed tube was heated for 20 hr at 100°. The tube was opened and the red solution was diluted to 100 ml with water. This solution was extracted with three 30-ml portions of ethyl acetate and then with three 30-ml portions of 2-butanol. The ethyl acetate layers

were combined and washed with ten milliliters of water. The solution was then evaporated to dryness to give 0.007 g of a yellow solid. The 2-butanol layers were combined and evaporated to dryness *in vacuo* to give 0.046 g of a red-brown solid. The aqueous and butanolic extracts were analysed by TLC and found to give essentially the same complex pattern of spots. In *t*-amyl alcohol:ethanol:concentrated ammonium hydroxide::80:7:20, the aqueous extract had at least 6 spots; a yellow spot that gave a brown color with ninhydrin was at R_F 0.15; other faint ninhydrin positive spots were at R_F values of 0.0, 0.39, 0.47, 0.56, 0.80. The spots at R_F 0.0 and R_F 0.43 were also Weber positive. In BAAmW, the ninhydrin positive DNP spot had an R_F value of 0.61; the ninhydrin positive DNP spot from the hydrolysis of DNP-peptide I had an R_F value of 0.60 on the same plate.

Stability of *bis*-DNP-β-lysine and *bis*-DNP-DAPA to Acid Hydrolysis

A sample of 0.023 g of *bis*-DNP-β-lysine in *ca.* five milliliters of 6 *N* hydrochloric acid was heated at 150° for six hours. The product was a yellow solution with a yellow precipitate. The precipitate was washed with ethyl acetate. The two solutions were analysed by TLC; the aqueous solution had one yellow, ninhydrin positive spot at R_F 0.74 in BAW; *bis*-DNP-β-lysine had a yellow spot at R_F 0.74. The ethyl acetate solution had a yellow, ninhydrin positive spot at R_F 0.76 in BW; *bis*-DNP-β-lysine had a yellow spot at R_F 0.76.

A sample of *bis*-DNP-DAPA in one milliliter of concentrated hydrochloric acid was heated in a pressure bottle on a steam bath for six hours

to give an orange-red solution with an orange-red precipitate. Analysis of the solution and the precipitate by TLC was carried out. In BW, the solution gave one yellow spot at an R_F value of 0.62; the precipitate gave one yellow spot at R_F 0.78, and bis-DNP-DAPA had an R_F value of 0.76. No ninhydrin positive spots were observed.

CHAPTER III

DISCUSSION OF RESULTS

At the beginning of this research, structures VIII and IX had been proposed for the structure of viomycin on the basis of degradation studies. The purpose of the present research was to carry our further degradation studies leading to the correct structure of viomycin.

Early in this research it was learned that the commercial viomycin that had been used previously in all of the degradative studies was not homogeneous. Attempts to find a system for the analysis of viomycin led to the use of silica gel HF_{254} TLC plates that had been partially deactivated with a pH 6 citrate buffer. When commercial viomycin was analysed using these plates in a buffer solvent, two components were observed as spots that fluoresced under UV light and gave purple colors with ninhydrin.

An attempt was made to separate these two components by fractional crystallization of their azo dye salts. The orange II and pHABS salts of viomycin were prepared. However, the intensity of the UV absorption at 268 mμ of the viomycin recovered from the dye salts was decreased by ca. 45 per cent. This was assumed to be due to partial degradation of viomycin by the strong sulfonic acid azo dyes during the reaction.

Gel filtration of commercial viomycin sulfate separated the two components so that material that was homogeneous by TLC and electrophoresis was obtained. A tall column of Bio Gel P-2 eluted with dilute formic acid was first used to separate the two components. The first

fractions from this column were composed of chromatographically homogeneous material, the minor component, that will be denoted V_{min}. The last fractions were chromatographically homogeneous material, the major component, that will be donated V_{maj}. The middle fractions were mixtures of V_{min} and V_{maj}. The same results were obtained when Sephadex G-15 was used, except that a slightly better separation was obtained. Further separation could be obtained by rechromatographing the mixtures. Commercial viomycin contains ca. 8-10 per cent V_{min}.

The gel filtration materials are composed of dextran chains which have been cross-linked to form "holes" of different sizes in the solid polymer. During a separation on the column, molecules of different sizes and shapes will diffuse into these holes. The larger molecules that do not fit into the holes very well are eluted faster than the molecules that can go into the holes. Thus the material acts as a molecular seive. That V_{min} is eluted from the column before V_{maj} means that its molecular weight, shape, or volume is greater than that of V_{maj}.

It was reported that capreomycin, an antibiotic that is very similar to viomycin, could be crystallized as its free base (31). The free base of V_{maj} was formed by anion exchange on a strongly basic anion exchange resin. When attempts were made to crystallize this material from water-ethanol-1-propanol, it was found that the free base gradually becomes insoluble in water, whereas viomycin sulfate was soluble in water at any pH.

The UV spectra of V_{maj} and V_{min} were found to be very similar with only slight differences between the extinction coefficients. Both

components had λ_{max}^{HCl} 269 mμ (ε, ca. 26,000); $\lambda_{max}^{H_2O}$ 269 mμ (ε, ca. 23,000-25,000); and λ_{max}^{NaOH} 290 mμ (ε, ca. 17,000).

The NMR spectra of V_{maj} and V_{min} both had a low field absorption around 2 τ. This absorption was either a doublet or a poorly resolved triplet (J = 3 cps) in the spectrum of V_{maj}. If this absorption was assumed (as done previously) (5) to represent one proton, then integration by planimeter of the remainder of the spectrum revealed a total of 45-48 protons. This absorption appeared as a singlet in the spectrum of V_{min}. There was an absorption at τ 3.6 (0.6 H, doublet, J = 8 cps) in the V_{maj} spectrum that was not present in the V_{min} spectrum. Other than these minor differences, the spectra were identical.

Both V_{maj} and V_{min} were hydrolysed, and the hydrolysate was analysed by an amino acid analyser. The values obtained indicated serine, β-lysine, DAPA, viomycidine and ammonia occur in the ratio 2:2:1:1:2 in both components. The standard sample for β-lysine was a sample of β-lysine·HCl; this material is very hygroscopic and an accurate weight would be very difficult to obtain. Thus a higher value would be obtained for β-lysine than was obtained in previous experiments (Table 2). The value for ammonia is also higher than that determined previously. An earlier amino acid analysis had urea and ammonia in the ratio 1:0.11 (9).

Perhaps the most accurate determination of the amino acid composition of viomycin was by a quantitative separation of all the hydrolysis components of viomycin on a tall Dowex-50 (H^+) column. These results are in Table 2. They show that serine, β-lysine, ammonia and DAPA were

isolated in the ratio 2:1:1:1. Since viomycidine was degraded by acid hydrolysis, and since different pathways existed which led to the formation of other guanidino compounds, only 0.4 mole of viomycidine per mole of viomycin was isolated (12).

Serine, DAPA, β-lysine, viomycidine and peptide III were identified as products of the vigorous acid hydrolysis of V_{maj} and V_{min}. Only faint traces of other ninhydrin and Weber positive compounds were detected. No urea was detected. A trace of a compound that gave an orange-yellow color with pDMAB reagent was also detected.

Previously it was reported that only faint traces of amino acids were observed when viomycin was subjected to hydrazinolysis. Either V_{maj} or V_{min} was allowed to react with hydrazine; the hydrazinolysate was allowed to react with benzaldehyde. The benzylidine derivatives that were formed were then extracted with ether or removed by filtration. Analysis of the aqueous layer by TLC showed a strong ninhydrin positive spot with the same color and R_F as serine in several systems. However, electrophoretic analysis of this solution showed that this compound was not serine, but a basic compound. This compound was isolated by preparative electrophoresis; it was found to have a UV absorption at $\lambda_{max}^{H_2O}$ 282 mμ ($E_{1\ cm}^{1\%}$, 103). The compound was hydrolysed overnight in 6 N hydrochloric acid; analysis of the hydrolysate by electrophoresis and TLC indicated that some viomycidine may have been released.

A second hydrazinolysis of V_{maj} was carried out and the UV spectrum of the hydrazinolysate was taken in acid and in base. There was a λ_{max}^{HCl} at 265 mμ with strong end absorption. The end absorption was

shifted to longer wavelength in base, but no discreet λ_{max} was observed.

The reaction of a peptide with anhydrous hydrazine gives cleavage of normal amide bonds to the corresponding carboxylic hydrazides and free amino groups. The UV chromophore of viomycin was not destroyed by hydrazinolysis. Any amide bonds between the chromophoric group and other amino acids should be cleaved. The basic compound that was isolated contained a chromophoric group and, since viomycidine was detected in its acid hydrolysis product, the viomycidine precursor in viomycin is probably involved in the chromophore of viomycin.

Johnson and co-workers reported that the chromophore of viomycin was destroyed at the same rate that urea was released during the hydrolysis of viomycin, and they concluded that urea was involved in the chromophore. They also concluded that the guanidino group was unlikely to be part of the chromophore (21). These conclusions are disproved by the presence of the chromophore in V_{maj} and V_{min}; no urea is obtained from the acid hydrolysates of these components. Analysis of the change in the UV spectrum of viomycin with pH showed that a group with a pKa of 12.4 was involved in the chromophore (4,6). Thus it is certain that urea is not involved in the chromophoric group, and that the guanidino group is part of the chromophore.

Viomycin was reported to give one mole of urea and desureaviomycin when it was heated in 0.1 N hydrochloric acid for six to eight hours. No urea could be detected when V_{maj} was heated in 0.1 N hydrochloric acid for six hours or even 18 hr. The composition of the product depended on the duration of the hydrolysis and on the way the product was isolated.

There were two components present in the hydrolysate. After six hours hydrolysis, there was still some unreacted viomycin. No unreacted viomycin was detected after 18 hr hydrolysis. The two components could be partially separated with a tall Sephadex G-15 column. The component that was eluted first from the Sephadex G-15 column had a $\lambda_{max}^{H_2O}$ at ca. 285 mμ and a λ_{max}^{NaOH} at 350 mμ; more of this component was produced with the longer hydrolysis time. The component eluted last had $\lambda_{max}^{H_2O}$ ca. 285 mμ These λ_{max} were estimated values since the peaks overlapped to some extent and changes in concentration shifted the observed λ_{max} slightly.

The elution pattern of these two components from the Sephadex G-15 column implied that the component with λ_{max}^{NaOH} 348 mμ had a greater molecular weight, shape, or volume than the component with λ_{max}^{NaOH} at ca. 285 mμ. This difference was very small, since there was considerable overlapping between the two components.

A sample of V_{maj} in 0.1 N hydrochloric acid was heated for eight hours and the hydrolysate was divided into two parts. One part was lyopholized and its UV spectrum was taken. The component with λ_{max}^{NaOH} at 350 mμ was the major component. The other half of the hydrolysate was isolated as done previously by partial neutralization to pH 5 using IR-45 (OH^-) followed by formation of the stoichiometric sulfate salt using an IR-45 ($SO_4^=$) column. The UV spectrum of this product showed that the component with a λ_{max}^{NaOH} at ca. 285 mμ was the major product.

Microorganisms that produce antibiotics are known to undergo mutations and to produce different compounds than the original antibiotic. A strain of S. griseus that was known to produce viomycin

10-15 yr ago was used recently in an attempt to prepare viomycin for biosynthetic studies (35). No viomycin was produced by this microorganism; it had evidently undergone a mutation. An antibiotic was produced which had λ_{max}^{HCl} and λ_{max}^{NaOH} at 267 mμ. No shift to longer wavelength was observed in alkaline solution. Another strain was obtained from the company that produces the presently available commercial viomycin, Charles Pfizer and Company. This strain produced a material that was identical to the commerical viomycin.

When labelled viomycin-^{14}C was produced by this strain using glucose-U-^{14}C as precursor to be used in biosynthetic studies in this laboratory, no urea was found in the acid hydrolysis products (12). Certainly even a trace of urea would have been detected, since the urea precursor in the viomycin molecule would be expected to be labelled to a detectable extent. All of the other hydrolysis products were significantly labelled.

No urea was obtained from V_{maj}, V_{min}, or the commercial viomycin used in this research when they were hydrolysed completely under the same conditions that urea had been obtained previously. At the beginning of this research a new supply of commercial viomycin was obtained. Since urea was reported as a component of viomycin when it was first discovered and in later work in this laboratory and in present work in other laboratories, it is evident that the previous structure elucidation has been done on urea-containing "old viomycin" and that "new viomycin" that does not contain urea was used in this research.

That another difference may exist between "old" and "new" viomycin

is indicated by the amount of ammonia released upon acid hydrolysis. The amino acid analyses of V_{maj} and V_{min} indicate two moles of ammonia per mole of viomycin. Analysis of "old viomycin" with an amino acid analyser gave 0.110 moles of ammonia per mole of urea (9).

An attempt was made to fit the analytical data of V_{maj} to the molecular formula of structure IX, $C_{23}H_{36}N_{12}O_8 \cdot 3/2\ H_2SO_4$ by adding water to the formula. A formula with 2.5 moles of water agreed with the analytical data. This formula has two serine units, one β-lysine unit, one DAPA unit, one viomycidine unit, one carbon dioxide unit and one urea unit. However, no urea was found among the hydrolysis products of V_{maj} or V_{min}. When one mole of urea is subtracted from the formula above and a molecule of water added to it to give $C_{22}H_{34}N_{10}O_8 \cdot 3/2\ H_2SO_4$, agreement with the experimental data is obtained by adding 1.5 moles of water of hydration to the formula. If ammonia is not a degradation product, an ammonia unit may be added and a water molecule substracted from the second formula to give $C_{22}H_{35}N_{11}O_7 \cdot 3/2\ H_2SO_4$. Agreement between the experimental per cent composition and that calculated for this formula is reached when 2.5 moles of water of hydration are added. These calculated per cent compositions are compared with those found for V_{maj} sulfate in Table 13. It is evident that the best agreement between a calculated composition and the experimental composition is for the formula where the urea is replaced by ammonia, the third formula. The C/N ratio, independent of the degree of hydration, is 1.69 for the experimental composition and 1.71 for the calculated composition. Thus the best molecular formula for V_{maj} sulfate is $C_{22}H_{35}N_{11}O_7 \cdot 3/2\ H_2SO_4 \cdot 5/2\ H_2O$.

Table 13. Comparison of Analytical Data for V_{maj}

Formula	Per Cent					Ratio
	C	H	N	O	S	C/N
Found	34.56	5.58	20.43	32.84	6.50	1.69
$C_{23}H_{36}N_{12}O_8 \cdot 3/2\ H_2SO_4 \cdot 5/2\ H_2O$	34.15	5.53	20.99	32.97	5.98	1.58
$C_{22}H_{34}N_{10}O_8 \cdot 3/2\ H_2SO_4 \cdot 3/2\ H_2O$	35.75	5.44	19.03	33.25	6.48	1.88
$C_{22}H_{35}N_{11}O_7 \cdot 3/2\ H_2SO_4 \cdot 5/2\ H_2O$	34.85	5.72	20.35	32.75	6.34	1.71

Johnson and co-workers had proposed VIII at the beginning of this research as the structure of viomycin; it has the urea unit joined to the rest of the molecule through a carbamate group. In order to investigate this proposed structural unit, samples of n-butylcarbamate, urea, and viomycin were allowed to stand at room temperature in 6 N hydrochloric acid and the carbon dioxide released was measured. One equivalent of carbon dioxide was released during four days hydrolysis from the n-butylcarbamate. No carbon dioxide was released in the hydrolysis of urea during three days hydrolysis. No carbon dioxide was released from viomycin during 16 days hydrolysis. Therefore there is no carbamate group in viomycin, since it would have been hydrolysed to some extend in 16 days.

Kellogg reported that if viomycin was allowed to stand in 6 N hydrochloric acid at room temperature for ten days, only serine and β-lysine are released. Serine readily reacted with two equivalents of

sodium periodate. In order to determine the amount of serine released during the room temperature hydrolysis, aliquots were taken from a solution of viomycin in 6 N hydrochloric acid over a period of 16 days and allowed to react with sodium periodate. The initial solution reduced 0.77 equivalents of sodium periodate. After 18 days, the hydrolysate reduced 2.38 equivalents of sodium periodate; 1.61 equivalents of sodium periodate in excess of the initial reaction were reduced. Thus there is about one equivalent of serine released in this hydrolysis. In addition to the release of serine, several changes took place in the UV absorption. The absorption at 268 mμ at pH 7 decreased to 8.5 per cent of its original value; the corresponding absorption at 275 mμ at pH 12 decreased to 20 per cent of its original value. A new λ_{max} at 297 mμ in the pH 7 buffer and 348 mμ in the pH 12 solution appeared; its intensity increased to a maximum $E^{1\%}_{1\ cm}$ of 106 (pH 7) and 83 (pH 12) after 1-2 days, and then decreased to ten per cent of its maximum value after 18 days. These changes corresponded to the changes in the UV spectrum when V_{maj} was heated in 0.1 N hydrochloric acid. Johnson has recently proposed XI as the viomycidine-DAPA precursor in viomycin itself. This compound would have a UV absorption of low intensity; only if the guanidino group were further conjugated could it be part of the chromophore of viomycin. Assuming the initial conjugation necessary for the chromophore of viomycin, loss of water from structure XI through dehydration could give a shift of the λ_{max} to longer wavelength through extension of the conjugation of the chromophore to give the absorption that appears after treatment with acid.

When the product of the 16 day room temperature 6 N hydrochloric acid hydrolysis was analysed by TLC, it was found to contain serine, β-lysine, peptide III and at least two unknown components. These two components could be purified by ion exchange chromatography or by gel filtration. The major component of the hydrolysate was named peptide I. The other unknown component was isolated in very low yield; it was called peptide II. No viomycidine or DAPA were found in the hydrolysate.

A partial separation of peptide I and peptide II was obtained using a Dowex-50 (H^+) column. Material enriched in peptide I was eluted from the column by water. This material was hydrolysed in 6 N HCl on a steam bath overnight to give a mixture of peptide III, viomycidine, DAPA, and serine. The Dowex-50 (H^+) column was eluted with 3 N hydrochloric acid; this eluate contained material that was enriched in peptide II. Acid hydrolysis of this material also gave peptide III, DAPA, viomycidine, and serine.

It was found that chromatographically homogenous peptide I could be obtained by chromatography of the gross hydrolysate over a Sephadex G-10 column followed by rechromatography of the first peak over a tall Sephadex G-15 column.

Peptide I was usually isolated as the sulfate salt. It was a white, amorphous solid that decomposed when it was heated above 200°C. It was moderately soluble in water. It decomposed when it was allowed to stand in water for several months.

Attempts to crystallize the sulfate and hydrobromide salts of peptide I from alcohol-water, acetone-water, and alcohol-acetone water mixtures failed.

The UV spectrum of peptide I indicated that it had a different chromophore than viomycin. Peptide I had λ_{max}^{HCl} at 294 mμ ($E_{1\ cm}^{1\%}$, 246) λ_{max}^{NaOH} at 291 mμ ($E_{1\ cm}^{1\%}$, 99) and 348 mμ ($E_{1\ cm}^{1\%}$, 158).

It is possible that peptide I may be a mixture of two very similar compounds. The major component has the chromophore with λ_{max}^{NaOH} at 348 mμ, and the other has the chromophore with λ_{max}^{NaOH} at ca. 285 mμ, corresponding to the two different chromophoric groups obtained earlier from the hydrolysis of V_{maj} with 0.1 N hydrochloric acid. These two compounds must be very similar structurally since they have the same electrophoresis mobility and TLC R_F value.

A sample of chromatographically homogenous peptide I was completely hydrolysed in acid. The hydrolysis products were found to be serine, DAPA, viomycidine, and peptide III.

The DNP derivative of peptide I was formed by standard procedures. End group analysis of peptide I by hydrazinolysis, and by hydrazinolysis and hydrolysis of DNP-peptide I revealed that its carboxyl terminal acid was serine and the β-amino group of DAPA was free. Also, peptide I gave the same basic compound upon hydrazinolysis that was obtained from V_{maj} or V_{min} upon hydrazinolysis. The β-DNP-DAPA derivative was synthesized for comparison by reaction of DNFB with the β-amino group of DAPA while the α-amino group was protected by the copper complex.

In an attempt to degrade viomycin to peptide fragments, viomycin was heated with a strongly basic ion exchange resin. The intensity of the UV absorption of the supernatant solution decreased gradually over 6 days to ca. 12 per cent of the original value. Analysis of the hydro-

lysate by TLC showed that β-lysine and serine were released after one day and that the intensity of the spots corresponding to serine and β-lysine increased through the sixth day. Analysis of the hydrolysate after six days showed that serine, β-lysine, and peptide III had been released. A spot with R_F 0.0 that was ninhydrin and Weber positive corresponded to unknown material. No spots corresponding to DAPA or viomycidine were observed.

The unknown material was isolated by ion exchange chromatography. The hydrolysate was chromatographed on IRA-400 (OH^-); the eluate from this column was passed directly over an IRC-50 (H^+) column. The acid eluate of the IRC-50 column contained the unknown material; this was the basic fraction. Chromatographically homogenous material could be obtained by rechromatographing the basic fraction over a larger IRC-50 (H^+) column and then a larger IRA-400 $(OH)^-$ column. This material gave serine, DAPA, viomycidine, and peptide III when it was completely hydrolysed in 6 N hydrochloric acid. The basic fraction could also be purified by gel filtration using a tall Sephadex G-10 column. This peptide from the resin hydrolysis of viomycin was found to be the same material as peptide I by TLC and electrophoresis.

It was reported (4, 5) that a mixture of guanidino compounds was obtained from the acid hydrolysate of viomycin. Viomycidine was the major component of this mixture. A minor component, referred to previously as "the lower R_F guanidino compound," has been isolated in greater yield. Floyd found that more of this compound could be obtained by hydrolysis of viomycin for six hours than for 16 hr (36). Larger

quantities of this compound, which will be referred to as peptide III, could be obtained using a Dowex-50 (H^+) column to separate the hydrolysis products of viomycin. The material obtained in this way was a mixture of at least three compounds, and peptide III was the major component. Peptide III was obtained as an amorphous, white, chromatographically homogenous sulfate or hydrochloride salt by chromatography of this mixture on an IR-45 ($SO_4^=$) or IR-45 (Cl^-) column followed by decolorization with Darco and further chromatography on a tall Sephadex G-10 column.

Peptide III·2 HBr was obtained from Peptide III hydrochloride by a standard procedure using IR-45 (Br^-) ion exchange resin. Peptide III·2 HBr was crystallized with difficulty from water-ethanol-n-butyl alcohol; it was recrystallized readily from acetone-water to give white needles. A yield of ca. 30% of crystalline material was obtained from ca. one gram of the crude mixture of peptide hydrobromides. An x-ray structure analysis is being carried out on the crystalline material. The crystal was orthorhombic, space group $P_{2_12_12_1}$. The crystal had cell dimensions of a = 8.17 ± 0.02 Å, b = 12.22 ± 0.02 Å and c = 15.34 ± 0.02 Å. The calculated density was 1.87, and the experimental density found by flotation was 1.87. The crystal has four peptide III·2 HBr·H_2O units per unit cell (37).

Johnson and co-workers isolated a compound they named viocidic acid (XII) as a degradation product of viomycin and determined its structure by an x-ray analysis. They found that the crystals of viocidic acid were orthorhombic, space group $P_{2_12_12_1}$; the cell dimensions were a = 8.17, b = 12.17, and c = 15.22 Å. The unit cell had

four units of $C_8H_{13}N_5O_2 \cdot 2\ HBr \cdot 3\ H_2O$. The bromine atoms were located from the Patterson function and the remaining atoms were located from electron density distributions. Fourier calculations gave the molecular structure. The R value converged to 0.121.

Since the cell dimensions of viocidic acid are very close to those of peptide III; it is evident that they are identical. The analytical data found for a sample of peptide III that had been dried to constant weight agreed very well with the formula $C_9H_{15}N_5O_3 \cdot 2\ HBr$ (403.136), whereas the formula reported by Johnson for viocidic acid was $C_8H_{13}N_5O_2 \cdot 2\ HBr \cdot 3\ H_2O$ (427.073), one carbon and one oxygen less and two waters more. The sample of peptide III lost 12.53 per cent of its weight when it was dried. A loss of 12.5 per cent water from peptide III corresponded to a loss of 3.3 water molecules during the drying process. A sample of peptide III that had been air dried only was analysed for C, H, N; the formula $C_9H_{15}N_5O_3 \cdot 2\ HBr \cdot 2.3\ H_2O$ agreed with this analysis. Another portion of this sample was dried to constant weight and analysed for C, H, N, and Br. The C/N ratio for this analysis was 1.39, different from the C/N ratio of the air dried sample (1.51). This indicated that one of the analyses was faulty. There was good agreement between the C/N ratio of the previous analysis on a dry sample (1.54) and the C/N ratio of the air dried sample. A loss of 8.03 per cent on drying corresponded to 2.1 water molecules. Evidently different amounts of water adhered to the crystals. No elemental analysis was given for viocidic acid. Calculation of the density of viocidic acid gave 1.87. The calculated and experimental

value for the density of the peptide III crystal is also 1.87. Calculated compositions for the different formulas are compared with experimental values in Table 14. A value that is independent of the dryness of the sample is the C/N ratio. The C/N ratio for $C_8H_{13}N_5O_2 \cdot 2$ HBr is 1.37. The C/N ratio for the $C_9H_{15}N_5O_3 \cdot 2$ HBr is 1.54. The observed C/N ratios are 1.54 (sample dried to constant weight) and 1.51 (sample air dried). From these data, peptide III cannot have the structure or formula of viocidic acid. The most reasonable formula for peptide III is $C_9H_{15}N_5O_3 \cdot 2$ HBr.

The UV spectrum of peptide III showed only end absorption when it was taken at a concentration ca. 20 times as great as is required for the chromophore of viomycin to be observed.

The NMR spectrum of peptide III·2 HBr was taken at room temperature and at 80°. Figure 5 is a composite of the two spectra; the HOD peak is not shown for either spectrum. All of the absorptions due to nonexchangeable protons at τ 4.5 (doublet), 5.2 (complex multiplet), and 6.0 (complex multiplet) had relative areas of 1.0:3.9:5.7, a total of 10-11 nonexchangeable protons.

The NMR spectrum of a sample of chromatographically homogenous peptide III hydrochloride was taken. This spectrum was found to be significantly different from that of the crystalline peptide III·2 HBr. There were additional absorptions at τ 6.4, 7.0 and 7.5 in the spectrum of peptide III hydrochloride. These absorptions were attributed to an impurity in the peptide III hydrochloride that was separated from it only by crystallization.

Table 14. Composition of Peptide III

Formula		Per Cent C	H	N	Br	Ratio C/N
Found[a]		26.46	4.54	17.35	39.72	1.53
Found[b]	1.	24.11	5.51	16.03	-----	1.51
	2.	24.80	4.67	17.87	37.75	1.39
$C_9H_{15}N_5O_3 \cdot 2\ HBr$		26.79	4.24	17.37	39.65	1.54
$C_9H_{15}N_5O_3 \cdot 2\ HBr \cdot H_2O$		25.65	4.54	16.64	-----	1.54
$C_9H_{15}N_5O_3 \cdot 2\ HBr \cdot 2\ H_2O$		24.65	4.82	15.95	-----	1.54
$C_9H_{15}N_5O_3 \cdot 2\ HBr \cdot 2.3\ H_2O$		24.30	4.89	15.75	-----	1.54
$C_8H_{13}N_5O_2 \cdot 2\ HBr$		25.76	4.05	18.72	42.84	1.37
$C_9H_{13}N_5O_2 \cdot 2\ HBr \cdot H_2O$		24.59	4.14	17.99	40.90	1.37
$C_8H_{13}N_5O_2 \cdot 2\ HBr \cdot 2\ H_2O$		23.57	4.21	17.25	39.18	1.37
$C_8H_{13}N_5O_2 \cdot 2\ HBr \cdot 3\ H_2O$		22.47	4.95	16.40	37.41	1.37

[a]This sample was dried to constant weight in vacuo at 25° C.

[b]This sample was air dried for analysis 1. Analysis 2 was made on the sample after it had been dried to constant weight.

A sample of peptide III hydrochloride was heated in 6 N hydrochloric acid on a steam bath for six hours. Peptide III was only partially degraded; the most intense spot in the TLC chromatogram corresponded to peptide III. The degradation products of peptide III were DAPA and viomycidine. The sum of the formulas of DAPA and viomycidine minus a mole of water is $C_9H_{15}N_6O_3$. Therefore one nitrogen atom in peptide III is common to DAPA and viomycidine depending on the mode of hydrolysis.

Peptide III formed an amorphous yellow DNP derivative when it was allowed to react with DNFB. An 88 per cent yield was obtained if a mono-DNP-peptide III was formed. The DNP-peptide III was purified further by chromatography on a Celite-silicic acid column to give chromatographically homogenous DNP-peptide III. DNP-peptide III was insoluble in most of the solvents tried except for DMF and DMSO. Attempts to crystallize it failed.

Hydrolysis of DNP-peptide III gave a mixture of at least six different components. One of the components had TLC R_F values that corresponded to β-DNP-DAPA. None of the other components were identified.

Johnson and co-workers reported as evidence for structure VIII that ε-DNP-β-lysine and β-DNP-DAPA are obtained from the hydrolysis of bis-DNP-viomycin. Mason and Kellogg found bis-DNP-β-lysine as the only DNP-derivative in the hydrolysis product of bis-DNP-viomycin. Partial degradation of bis-DNP-β-lysine was obtained when the compound was heated in 6 N hydrochloric acid, since ninhydrin positive material was

produced. These results corresponded to those of Kellogg, who found that hydrolysis of bis-DNP-viomycin in 6 N hydrochloric acid released some 2,4-dinitrophenol, whereas hydrolysis in concentrated (ca. 12 N) hydrochloric acid in a sealed container gave only bis-DNP-β-lysine.

It was further found that bis-DNP-DAPA was stable when it was heated at steam bath temperature in concentrated hydrochloric acid under pressure. Thus, if any DNP-DAPA had been formed in the hydrolysis of bis-DNP-viomycin, it should have been detected.

CHAPTER IV

CONCLUSIONS

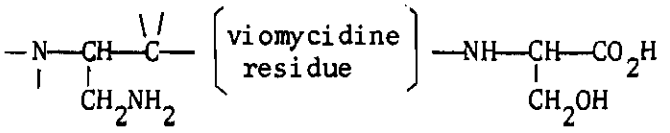
The present commerically available viomycin does not release urea upon acid hydrolysis as does the "old viomycin" used in research which has been reported previously. The "new viomycin" can be separated into two chromatographically homogenous components by gel filtration. The minor component, V_{min}, comprises about 8-10 per cent of the mixture. It is differentiated from the major component, V_{maj}, only by TLC and gel filtration. The two components have the same UV, NMR, and IR spectra; they have the same hydrolysis products in the same ratio.

The new viomycin gives a basic compound upon hydrazinolysis that releases viomycidine upon acid hydrolysis. The UV chromophore of viomycin does not result to any extent from a urea residue. The chromophore is related to the guanidine group in viomycin.

When viomycin is allowed to stand in acid or is heated in dilute acid, the UV absorption is shifted to longer wavelength and a mixture of compounds results. The chromophore is completely destroyed by further treatment with acid.

Peptides I and II, serine, and β-lysine are the major components of the mixture that is obtained when viomycin is allowed to stand in 6 N hydrochloric acid for 15-20 days. No carbon dioxide is released. Peptides I and II give serine, DAPA, viomycidine, and Peptide III upon further hydrolysis. Serine is the carboxyl terminal amino acid of

peptide I, and the β-amino group of DAPA is free. A partial structure for peptide I can be drawn as structure XIV. Peptide I contains a



XIV

chromophoric group that is closely related to viomycin. Peptide I is similar to Peptide B, isolated by Kitagawa and coworkers. Peptide B contains serine, β-lysine, viomycidine and DAPA (2:1:1). It has a UV absorption at 268 mμ, and the β-amino group of DAPA is free. The amino acid sequence of peptide B was shown to be β-lysylserylDAPAylviomycidylserine. If β-lysine is cleaved from this molecule, a serylDAPAylviomycidylserine unit is left. The experimental data do not preclude an N-terminal serine for peptide I. Thus the partial structure for peptide I supports the peptide sequence XIII proposed for viomycin by Kitagawa.

Peptide III is a guanidino compound that occurs in the acid hydrolysates of viomycin, peptide I, and peptide II. It forms a crystalline dihydrobromide ($C_9H_{15}N_5O_3 \cdot 2$ HBr) that has the same space group and unit cell dimensions as a compound isolated by Johnson that he called viocidic acid. Structure XII ($C_8H_{13}N_5O_2$) was assigned to viocidic acid on the basis of an x-ray analysis of its crystalline hydrobromide ($C_8H_{13}N_5O_2 \cdot 2$ HBr$\cdot 3$ H_2O). Peptide III and viocidic acid evidently are the same compound. Thus there is some doubt that structure XII is the correct structure of viocidic acid. An x-ray analysis of peptide III$\cdot 2$ HBr is in progress; it should resolve this doubt.

Viomycidine and DAPA were obtained in rather low yield when peptide III was hydrolysed in acid. Since the sum of the formulas of viomycidine and DAPA has six nitrogen atoms and only five nitrogen atoms are contained in peptide III, then one nitrogen atom is common to DAPA and viomycidine. Peptide III forms a mono-DNP derivative that gives β-DNP-DAPA upon hydrolysis. The data for peptide III gives further support to the proposed DAPAylviomycidine unit in XIII.

None of the data obtained is in conflict with structure XIII proposed as the peptide sequence of viomycin by Kitagawa except that urea is not found in the viomycin used in this research.

The structural unit of viomycin that is still undefined is the ultraviolet chromophore. An x-ray analysis of the structure of peptide I should lead to the structure of the chromophoric group. Peptide I is the smallest unit yet obtained from viomycin that still shows any UV absorption; thus it would be the most suitable compound for x-ray analysis of the chromophoric group. Another possible route to the chromophore structure is the hydrazinolysis product of viomycin. The component of the hydrazinolysate that has the chromophore of viomycin should be isolated. Determination of its structure may also lead to the structure of the chromophore.

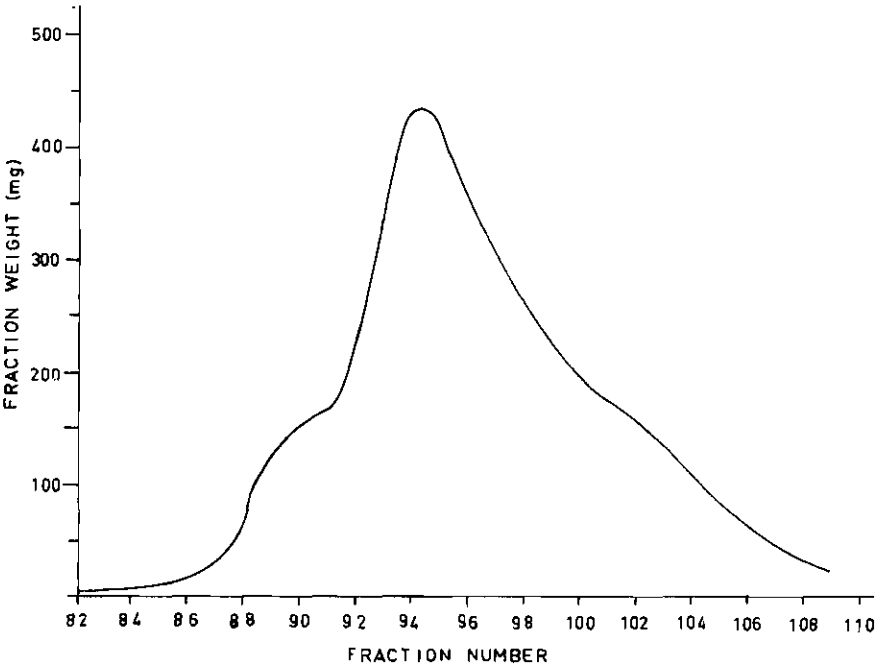


Figure 1. The Chromatogram of the Gel Filtration of Viomycin Using Bio Gel P-2.

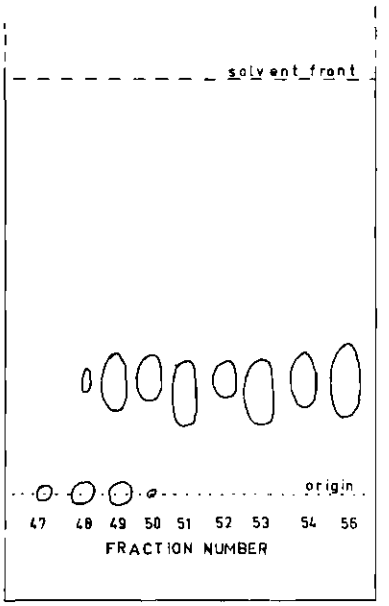


Figure 2. TLC of Fractions from the Sephadex G-15 Chromatography of viomycin.
CB plate, CB solvent, developed two times
UV light visualization

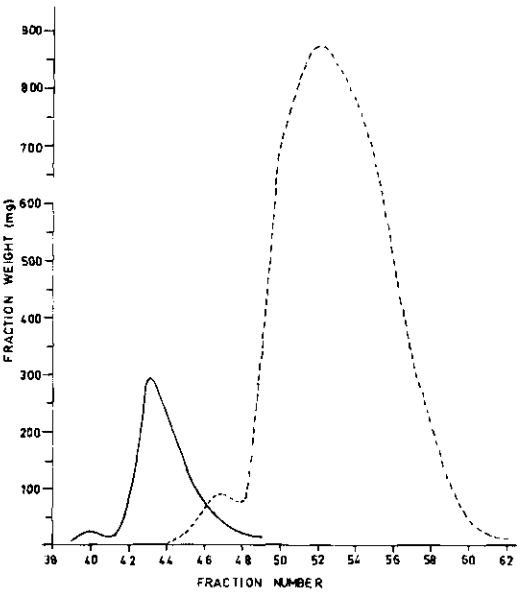


Figure 3. The Chromatogram for the Gel Filtration of Viomycin Using Sephadex G-15.
550 cm column ———
220 cm column -----

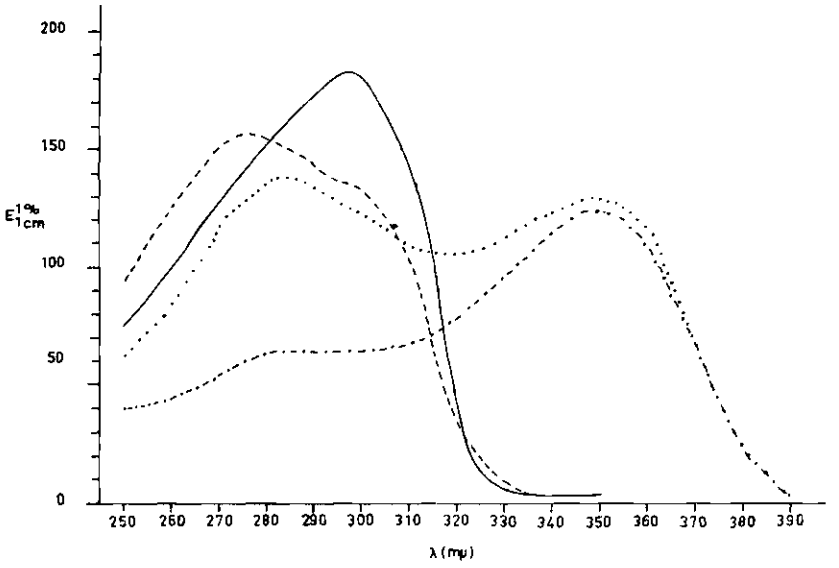


Figure 4. The UV Spectra of the Mild Acid Hydrolysis Products of Viomycin with and without an Ion Exchange Workup.
Lyophilized sample: in acid ———; in base ----
Resin Workup: in acid ----; in base ----

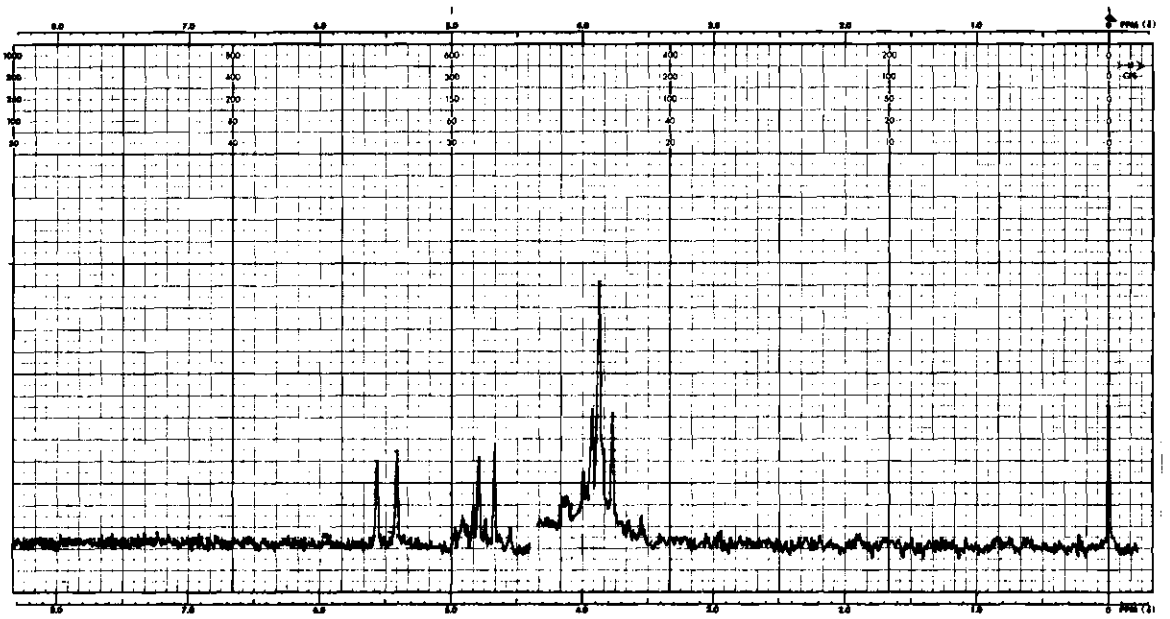


Figure 5. The NMR Spectrum of Peptide III.

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