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THE CHEMISTRY AND BIOSYNTHESIS OF VIOMYCIN

A THESIS

Presented to

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GLOSSARY OF ABBREVIATIONS

AES	automatic external standardization
cpm	counts per minute
DAPA	α,β-diaminopropionic acid
dpm	disintegrations per minute
DSS	sodium 2,2-dimethyl-2-silapentane-5-sulfonate
E	counting efficiency
ir	infrared (spectroscopy)
m	multiplet
MHz	megahertz
nmr	nuclear magnetic resonance (spectroscopy)
POPOP	1,4-bis[2-(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole
psig	pounds per square inch gauge
s	singlet
STP	standard temperature and pressure
tlc	thin layer chromatography
tT2l	toluene-Triton X-100 (2:1) scintillation solution
U-^{14}C	uniformly labeled with carbon-14
uv	ultraviolet (spectroscopy)

SUMMARY

Viomycin yields upon complete acid hydrolysis *L*-serine, *L*-α,β-diaminopropionic acid, *L*-β-lysine (*L*-3,6-diaminohexanoic acid), carbon dioxide, ammonia, and a strongly basic amino acid named viomycidine (2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid). The purposes of this research were twofold: first, to continue the investigations into the biosynthetic pathways leading to viomycin by studying several suspected biological precursors of the amino acids and the other components in viomycin; second, to further investigate the chemistry of the intact viomycin molecule.

Samples of radioactively labeled viomycin were produced by the addition of ^{14}C-labeled compounds suspected of being precursors of one or more of the amino acid residues in viomycin to the growth medium of *Streptomyces griseus* var. *purpurea*. These samples of viomycin were then hydrolyzed, and the hydrolysis components were separated by ion-exchange chromatography. The activities of the hydrolysis components were determined, and certain of the hydrolysis components that contained significant activity were degraded chemically in order to obtain data concerning the labeling patterns of those components.

DL-Arginine-amidine-^{14}C, *DL*-ornithine-1-^{14}C, *DL*-arginine-1-^{14}C, *DL*-arginine-5-^{14}C, *L*-α,β-diaminopropionic-U-^{14}C acid, *L*-β-lysine-^{14}C (labeling pattern undetermined), and *L*-viomycidine-^{14}C (labeling pattern undetermined) were investigated as possible precursors for viomycin biosynthesis. It was found that the arginine-amidine-^{14}C, arginine-1-^{14}C,

and arginine-5-^{14}C were incorporated directly into viomycidine with no scrambling of the labeling pattern. The ornithine-1-^{14}C was found to be incorporated into viomycidine to a large extent, but it would appear that some scrambling of the labeling pattern occurred.

D-Glucose-U-^{14}C was used as a precursor in order to produce a source of ^{14}C-labeled α,β-diaminopropionic acid, β-lysine, and viomycidine. The α,β-diaminopropionic-U-^{14}C acid was used as a precursor for viomycin biosynthesis. It was found that only the α,β-diaminopropionic acid residue of this viomycin sample was labeled and thus, α,β-diaminopropionic acid is not a precursor for the other amino acids.

Similar results were found for the viomycin produced from the β-lysine-^{14}C (labeling pattern undetermined). Only the β-lysine from this viomycin was labeled, showing that β-lysine is not a precursor for the other amino acids.

The viomycidine-^{14}C (labeling pattern undetermined) was not incorporated to a significant extent into any of the amino acid residues of viomycin. This was further evidence that viomycidine does not occur *per se* in the viomycin molecule, but rather is a product of the acid hydrolysis of viomycin.

Lysine-^{15}N, serine-^{15}N, and 3,3-dideuterioserine were also studied as precursors for viomycin biosynthesis. By use of low resolution mass spectrometry, it was found that both nitrogen atoms of lysine were incorporated into β-lysine without prior conversion to free ammonia. Serine was also found to contain nitrogen from the lysine precursor; the α,β-diaminopropionic acid was found to contain ^{15}N at

both the α- and the β-positions, suggesting that α,β-diaminopropionic acid is produced by amination of a serine residue involving a second serine molecule as the nitrogen donor.

From the serine-^{15}N precursor, the nitrogen of serine and both nitrogen atoms of α,β-diaminopropionic acid were found to be labeled. Only the β-nitrogen atom of β-lysine was found to contain ^{15}N-labeling. These results are in agreement with those of the previous experiment.

3,3-Dideuterioserine was used as a precursor in viomycin biosynthesis. Although the mass spectral data were of low precision, serine and diaminopropionic acid both appeared to retain the two deuterium atoms at the C-3 position, suggesting that the proposal of aldehyde formation at C-3 of serine prior to amination is incorrect.

It was found that viomycin was catalytically hydrogenated in 50 per cent aqueous acetic acid using a 10 per cent platinum on carbon catalyst. Sephadex chromatography of the perhydro product revealed this material to be 93 per cent homogeneous. Hydrolysis of perhydroviomycin, followed by ion exchange chromatography of the hydrolysate, showed *L*-serine, *DL*-alanine, ammonium chloride, *L*-α,β-diaminopropionic acid, *L*-β-lysine, capreomycidine [α-(2-iminohexahydro-4-pyrimidyl)glycine], and carbon dioxide as the major components. The serine to alanine ratio was found to be 2:1, indicating that alanine is not produced from serine by hydrogenolysis of the C-3 hydroxyl group. The pre-viomycidine unit in viomycin was converted into capreomycidine during the hydrogenation process.

The perhydroviomycin was found to possess no uv chromophore, but bioassay revealed that the perhydroviomycin was approximately 30 per cent as active as commercial viomycin.

CHAPTER I

INTRODUCTION

The Chemistry of Viomycin

Viomycin is a broad spectrum antibiotic that was isolated in 1950 simultaneously by Charles Pfizer and Company[1] and Parke, Davis and Company[2] from cultures of *Streptomyces puniceus* and *Streptomyces floridae*. While early studies indicated that viomycin was particularly effective against *Mycobacterium tuberculosis*,[1] later clinical studies revealed that kidney damage, vestibular dysfunction, electrolyte imbalance, and hypersensitivity resulted from extended use.[3] In spite of these rather serious toxic reactions, viomycin is still used clinically in cases where the tuberculosis microorganism has become resistant to streptomycin.

Analysis of the crystalline sulfate, hydrochloride, picrate, and Reineckate salts of viomycin indicated an empirical formula of $C_{18}H_{31\text{-}33}N_9O_8$ for the free base.[3,4] The sulfate salt has a specific rotation of -39.8° in water[2] and melts at 252° with decomposition. A molecular weight of 772 was indicated for the sulfate salt from diaphragm-cell diffusion studies. This, together with other analytical data and chemical evidence, suggested a molecular formula of $C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$ (mol wt 836).[5] Summation of all known hydrolysis fragments gave a molecular formula of $C_{20}H_{36}N_{12}O_7 \cdot 3/2\ H_2SO_4$ (mol wt 707.73).[6] An average of six separate analyses of viomycin

sulfate gave the following per cent composition: C, 35.71; H, 5.83; N, 21.19; S, 5.51; C/N ratio, 1.69.

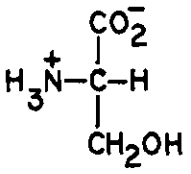
Viomycin is a strong base and has pK_a values of 8.3, 10.3, and 12.2 in water. The absence of a pK_a value lower than 8.3 indicates that viomycin contains no free carboxyl group.[5] Van Slyke primary amino nitrogen determinations indicated that 1.22 primary amino groups in viomycin react in 2.5 min, 1.98 groups react in 15 min, and 2.16 groups react in one hour (based on an assumed molecular weight of 836).[7] Viomycin gave positive Sakaguchi and ninhydrin tests, but negative Benedict's, Fehling's, and maltol tests.[1,2,7] These results suggested the presence of guanidino and amino groups. The fact that viomycin gave a positive biuret test[4] together with its resistance to mild acid hydrolysis indicated that viomycin had a peptidic function. On the basis of color tests and the failure of viomycin to react with periodic acid, the presence of a carbohydrate grouping appeared unlikely.

The ultraviolet spectrum of viomycin consists of one strong absorption, the position and intensity of which are dependent on the *p*H of the solution. This absorption appears at 268 nm (ε, 23,300) in 0.1 *N* hydrochloric acid, at 268.5 nm (ε, 22,900) in neutral solution and at 282.5 nm (ε, 14,600) in 0.1 *N* sodium hydroxide. Determination of the ultraviolet spectra in solutions of *p*H 10, 11, 12, 13, and 14 indicated two isosbestic points at 235 and 281 nm, suggesting that one dissociating group was involved with the chromophore.[5,8] It was also shown that the group involved had a pK_a value of 12.4, which indicated that

the guanidino group present in viomycin was involved in the ultraviolet chromophore.[5] When viomycin was heated in 0.1 *N* hydrochloric acid at 100°, the ultraviolet absorption gradually decreased and disappeared entirely after six hours. A lyophilized sample of this solution showed an ultraviolet absorption, however.

The nmr spectrum of viomycin sulfate in deuterium oxide showed absorptions at τ 1.93 (s), 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.[7] The infrared spectrum of viomycin sulfate as a potassium bromide pellet showed very broad absorptions of an indistinct nature, and no useful information has been derived from it.

Hydrolysis of viomycin sulfate using 1 *N* hydrochloric acid at 37° for 11 days released no amino acids, but the biological activity was reduced by 75 per cent.[4] Vigorous acid hydrolysis using 6 *N* hydrochloric acid at 100° for 20 hr destroyed the ultraviolet chromophore completely and amino acids were released.[4] Carbon dioxide, ammonia, and urea were identified in the hydrolysis mixture. Four other major products, all ninhydrin positive, were isolated from the hydrolysis mixture by using cation exchange chromatography with Zeo Rex (H^+).[4] The four ninhydrin positive products were shown to be *L*-serine (1), *L*-α,β-diaminopropionic acid (2), *L*-β-lysine (*L*-3,6-diaminohexanoic acid) (3), and a mixture of guanidino compounds in the ratio 2:1:1:1. *L*-serine was identified by optical rotation, analysis, preparation of the *N*-2,4-dinitrophenyl derivative, and by the identity of the infrared absorption spectrum with that of authentic *L*-serine.[4]



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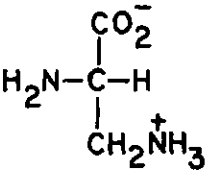

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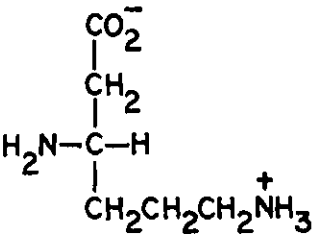

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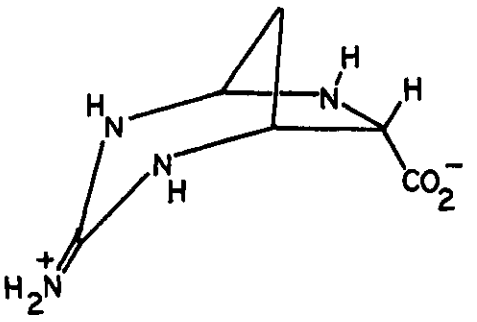
L-α,β-Diaminopropionic acid was identified as the monohydrochloride salt by analysis, van Slyke amino nitrogen determination, and ninhydrin carbon dioxide determination. The *L*-configuration was indicated for α,β-diaminopropionic acid on the basis of its optical rotation and the optical rotation of the *N,N'*-dibenzoyl derivative.[5] Analyses of the crystalline hydrochloride, sulfate, picrate, and *p*-hydroxyazobenzene-*p'*-sulfonate salts of *L*-β-lysine showed that this compound was identical with the isomer of lysine previously isolated from streptolin and streptothricin hydrolysates, which was shown to be identical with synthetic *L*-3,6-diaminohexanoic acid.[4,9]

The mixture of guanidino compounds was found to have one major component that was isolated in crystalline form and named viomycidine.[10,11] A single crystal X-ray diffraction analysis of viomycidine hydrobromide revealed that 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid (4) is the correct structure for viomycidine.[6,12,13]

One of the minor guanidino components is closely related to viomycidine, and has been named isoviomycidine.[14] This compound gave positive ninhydrin and Weber tests, and was found to be very similar in chemical properties to viomycidine. It was believed to be an isomer of



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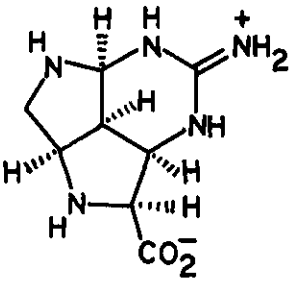
viomycidine. Isoviomycidine and viomycidine were separated by chromatography of a mixture of ^{14}C-labeled compounds over a long Dowex-50(H^+) ion exchange column; isoviomycidine was found to be slightly less basic than viomycidine. The isoviomycidine fractions were then pooled and again chromatographed over the Dowex-50(H^+) column. The purity obtained by this method was determined by liquid scintillation counting of the ^{14}C content of each fraction from the column. This purified material was converted to both the stoichiometric hydrochloride and hydrobromide salts, but neither salt could be crystallized.[14] The nmr spectrum of the purified material showed absorptions at τ 4.29-4.75(multiplet), 5.60(multiplet), and 7.05-8.33(complex multiplets), and was quite different in appearance from the spectrum of viomycidine.[6] No structure has yet been derived for isoviomycidine.

One other guanidino compound was isolated from the acid hydrolysate of viomycin and was named viocidic acid ($C_8H_{13}N_5O_2 \cdot 2HBr \cdot 2H_2O$). Viocidic acid was subjected to a single crystal X-ray diffraction analysis, which revealed the structure to be 2,5,8,10-tetraaza-9-

iminotricyclo[5.3.1.$0^{4,11}$]undecane-6-carboxylic acid (5).[13,15]



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Basic hydrolysis of viomycin with 0.5 *N* lithium hydroxide or 0.43 *N* barium hydroxide at 95° for three days yielded carbon dioxide, ammonia, and a number of amino acids. These amino acids were identified by two-dimensional paper chromatography as β-lysine, serine, α,β-diaminopropionic acid, and alanine.[5] Since alanine was not found in the acid hydrolysate of viomycin, the basic hydrolysate was subjected to preparative paper chromatography, and a quantity of the alanine was isolated and positively identified as *DL*-alanine.[16] The presence of alanine in the basic hydrolysis of viomycin and its absence from the acid hydrolysate was attributed to the attack of base on serine to give alanine. It has been shown that treatment of serine under the same conditions used for the basic hydrolysis of viomycin yielded a mixture of serine and alanine.[16]

Partial acid hydrolysis of viomycin gives four peptides.[17,18] Peptide A showed positive ninhydrin and Sakaguchi reactions, pK_a values of 9.9 and 11.2, and gave ultraviolet absorptions similar to viomycin, λ_{max} 275 nm (ε, 5100) at *p*H 1 and λ_{max} 295 nm (ε, 3600) at *p*H 10.

Hydrolysis of Peptide A with 12 *N* hydrochloric acid gave α,β-diaminopropionic acid and viomycidine in a 1:1 molar ratio. Hydrolysis of the 2,4-dinitrophenyl derivative of Peptide A gave bis-2,4-dinitrophenyl-α,β-diaminopropionic acid.[18] Peptide B gave equimolar amounts of β-lysine and serine on complete hydrolysis, and 2,4-dinitrophenylation of Peptide B, followed by hydrolysis, gave mono-ε-2,4-dinitrophenyl-β-lysine and *N*-2,4-dinitrophenylserine.[17] Peptides C and D were only isolated from hydrolysis of viomycin with 0.1 *N* lithium hydroxide in low yield. Peptide C was reported to give equimolar amounts of β-lysine and viomycidine, and Peptide D gave equimolar amounts of β-lysine, viomycidine, and serine upon complete hydrolysis.[17]

Kitagawa and coworkers[19] also isolated a series of peptides through partial hydrolysis of viomycin. Peptide A was separable into three substances, each of which had a peptide sequence of β-lysylseryl-diaminopropionylviomycidylserine, where no amino group of α,β-diaminopropionic acid was free. Peptide B was found to be β-lysylseryldiaminopropionyl(free β-NH_2)viomycidylserine, and Peptide C was seryldiaminopropionyl(free β-NH_2)viomycidine. From these data and the fact that Peptide A_{III} gave urea upon mild acid hydrolysis, they suggested that the amino acid sequence of viomycin was β-lysylseryldiaminopropionyl-(no free NH_2)viomycidylserylurea.

Oxidation of viomycin by treatment with aqueous potassium permanganate, bromine-water, or ozone (hydrogen peroxide workup) destroyed the ultraviolet chromophore, but no difference was noted between the composition of the acid hydrolysate of the oxidation product and the

composition of the usual viomycin acid hydrolysate.[5,7] Johnson and coworkers, however, have reported that oxidation of viomycin with potassium permanganate under undefined conditions led to formylurea and oxalic acid.[20]

An interesting ambiguity has arisen during the chemical studies on viomycin. 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 to 15 years ago was used recently in an attempt to prepare viomycin for biosynthetic studies.[21] No viomycin was produced by this microorganism; instead, an antibiotic was produced that had an absorption in the ultraviolet spectrum at 267 nm in both acid and alkaline solution. Another strain of *S. griseus* was obtained from Charles Pfizer and Company, which produces all of the presently available commercial viomycin. This strain produced a material that was identical to commercial viomycin.

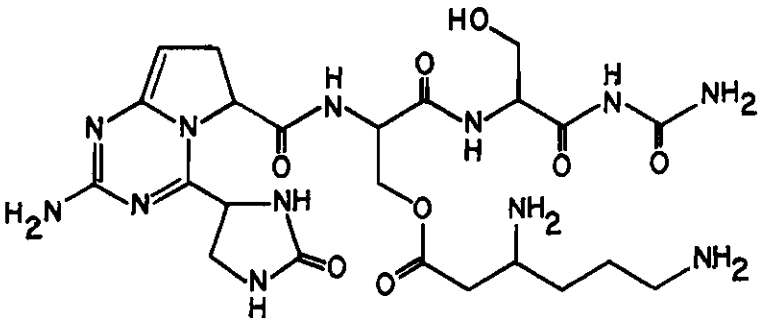
When labeled viomycin-^{14}C was produced by this strain from the precursor glucose-*U*-^{14}C, no urea was found in the acid hydrolysate.[6] Even a trace of urea would have been detected, since the urea precursor in the viomycin molecule would be expected to be labeled to a detectable extent. All of the other hydrolysis products were significantly labeled.

Beginning with a new batch of commercial viomycin sulfate received about 1965, no urea has been found in acid hydrolysates from any samples of commercial viomycin sulfate received since that time. Urea

was reported as a component of viomycin when it was first discovered, in early work in this laboratory, and in present work in other laboratories. It is evident that the previous work on viomycin has been done on urea-containing "old viomycin" and that the present-day commercial viomycin is a "new viomycin" that does not contain a urea residue. No other differences in behavior have been noted between "new" and "old" viomycin. Several recent literature sources[13,19,20] report finding urea under many different degradative conditions; it is therefore evident that these workers are still using the "old viomycin" for their studies on viomycin.

Several structures have been proposed for viomycin, each of which is incorrect in light of the currently accepted data about viomycin. In 1965, Dyer and coworkers proposed structure 6 as the structure of viomycin.[11] Kitagawa and coworkers reported the amino acid sequence is as is shown in structure 7.[19] Polish workers have suggested yet another structure (8), which does not seem to fit a large part of the existing data available on viomycin.[22] Perhaps the best structure for viomycin presented so far was proposed by Johnson and his coworkers and is shown as structure 9.[13]

None of these structures can apply to "new viomycin," since they all contain a urea residue. Structure 6 is no longer considered valid due to the fact that it has been found that the viomycidine residue obtained from the acid hydrolysate does not exist as such in the intact molecule. Structure 7 was not proposed as a complete structure, but only as an amino acid sequence. Structure 8 has no ultraviolet



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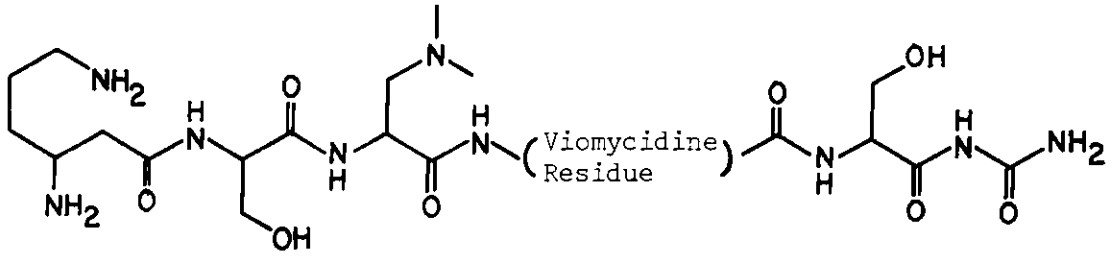

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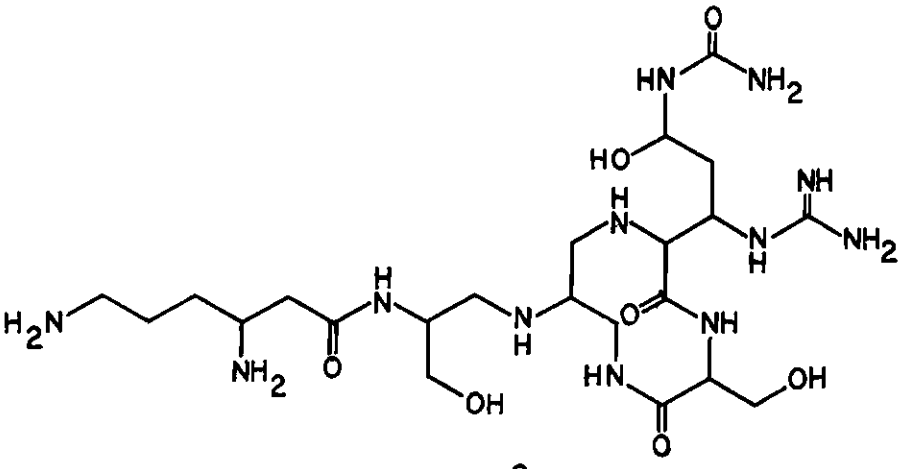

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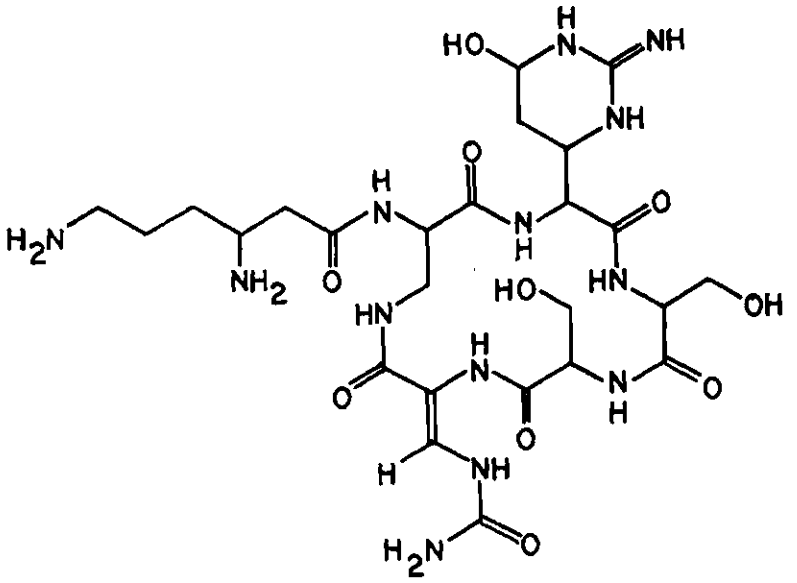


9

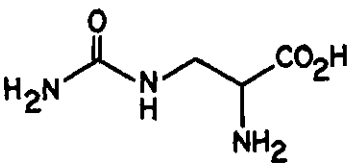
chromophore and it is not readily apparent how viomycidine could arise under the conditions of acid hydrolysis. Although structure 9 contains a urea residue, most of the other data on viomycin is consistent with this structure, at least for "old viomycin." Simple removal of the carbamoyl group ($-\overset{O}{\overset{\|}{C}}NH_2$) would result in a vinylamine system, which is known to be hydrolyzed very rapidly in aqueous solution. For this reason, "new viomycin" apparently differs from structure 9 by more than just the urea residue.

The Biosynthetic Studies of Viomycin

Although little direct information is available concerning the biosynthesis of the intact viomycin molecule, some information is available on the biosynthesis of the various hydrolysis products of viomycin.

Since *L*-serine is one of the common amino acids, it should be available to the organism producing viomycin either from the growth medium or by well-known biosynthetic pathways[23] and could be incorporated intact into the viomycin molecule.

α,β-Diaminopropionic acid and several of its derivatives have been isolated from natural sources. Albizzine (α-amino-β-ureidopropionic acid, 10) has been isolated from several species of *Mimosaceae*,[24]



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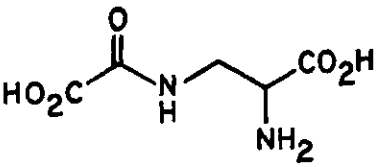
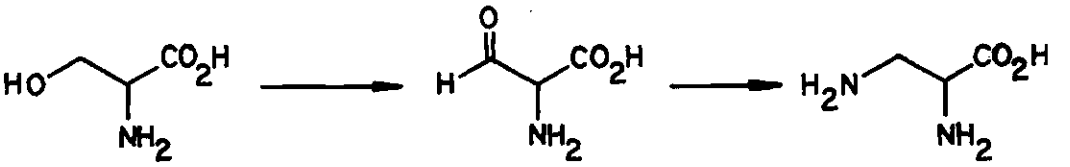


11

and β-*N*-oxalyl-α,β-diaminopropionic acid (11) has been isolated from *Lathyrus sativus*.[25] *L*-α,β-Diaminopropionic acid has been isolated in high concentration from seeds of *Vicia baicalensis*[26] and also from the hydrolysate of the antibiotic edeine.[27] *D*-α,β-Diaminopropionic acid has been found in the digestive fluid of the fifth instar of the larvae of *Bombyx mori*.[28]

The biosynthesis of albizzine has been studied in *Albizzia loparetha* by Reinbothe.[29] Glycine-1-^{14}C, glycine-2-^{14}C, serine-1-^{14}C, and serine-3-^{14}C were incorporated into albizzine to a significant extent. Formaldehyde-^{14}C and glyoxylate-U-^{14}C were incorporated to some extent, probably via serine. Uric acid-2-^{14}C was incorporated slightly, but carbon dioxide-^{14}C, formic acid-^{14}C, urea-^{14}C, uricil-

2-^{14}C, and arginine-amidine-^{14}C gave only negligible incorporation. Reinbothe suggested the pathway in Equation 1 for the biosynthesis of α,β-diaminopropionic acid.[29]



(1)

In studies done in these laboratories, 3,3-dideuterioserine was incorporated into viomycin. Preliminary low resolution mass spectrometry indicated that the α,β-diaminopropionic acid that was found in the acid hydrolysate had two deuterium atoms.[14] This would indicate that serine is aminated directly by displacement. This evidence does not, however, necessarily exclude that the oxidation process is not also operative as a possible pathway from serine to α,β-diaminopropionic acid.

L-β-Lysine (3,6-diaminohexanoic acid) has been derived by acid hydrolysis from a number of antibiotics other than viomycin, including streptolin AB,[30] streptothricin,[31] roseothricin,[32] and geomycin.[33] Studies of *L*-lysine fermentation by an unidentified species of *Clostridium* revealed that *L*-β-lysine accumulated by deletion of the cofactors (nicotinamide-adenine dinucleotide and adenosine-5'-diphosphate) required for lysine fermentation.[34] It was also found that β-lysine is formed from lysine by a readily reversible reaction, that it is fermented more

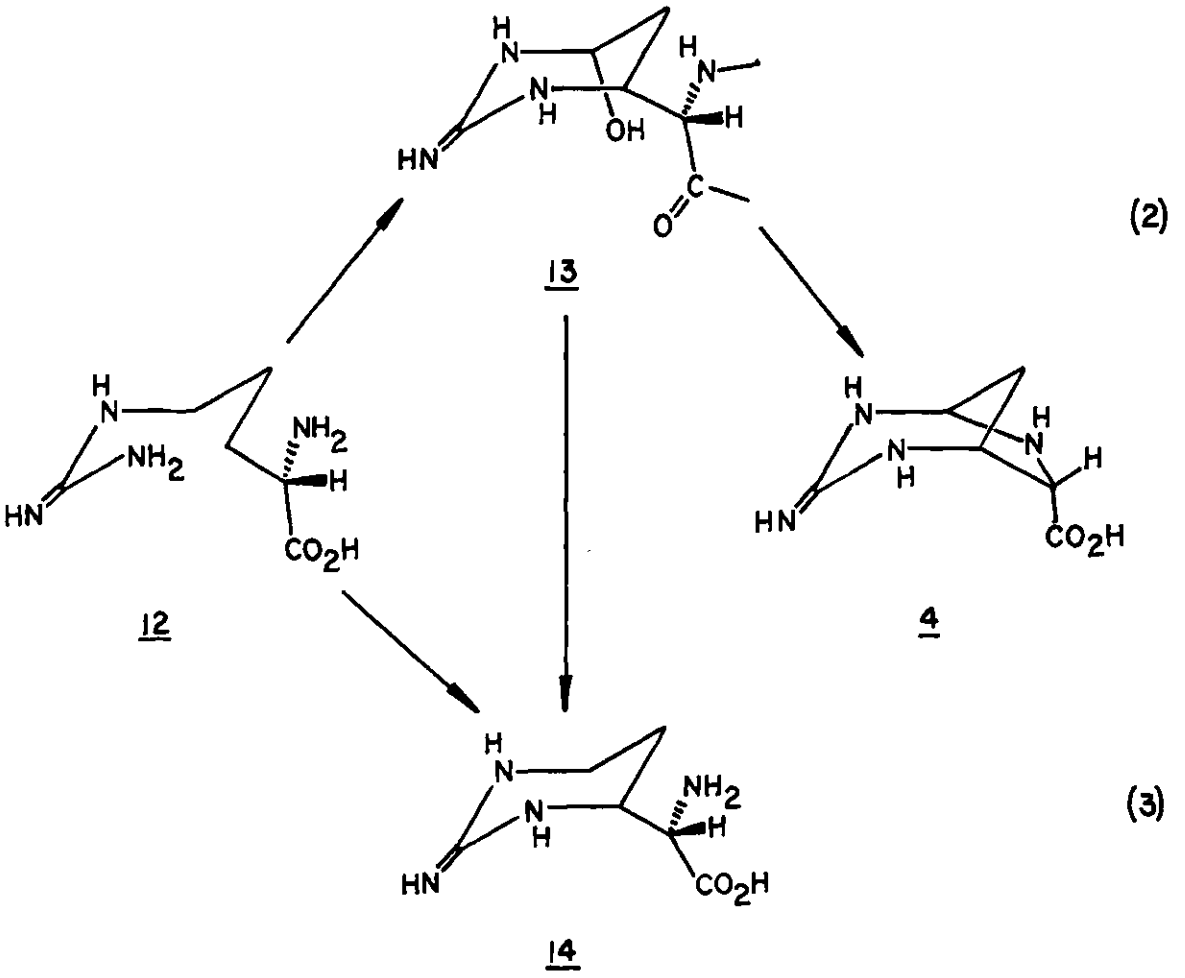
rapidly than lysine when the suitable cofactors are added to the culture medium, and that the addition of lysine does not markedly inhibit β-lysine fermentation. It was concluded that β-lysine lies on or very close to the path of lysine fermentation to ammonia and acetic and butyric acids. It was found that α-ketoglutarate and coenzyme A were required cofactors in the conversion of lysine to β-lysine.[34] In a later series of experiments using *Clostridium sticklandii*, it was shown that the fermentation of lysine to acetic acid, butyric acid, and ammonia involves two successive amino group migrations, first from the α to the β position to form β-lysine, then from the ε to the γ position to give 3,5-diaminohexanoic acid.[35] It was found that a cobamide enzyme was required for the second conversion, but not the first, which apparently requires pyridoxal phosphate instead.[36] In addition, it was shown that both the migration of the α-amino group to the β-position, and the migration of the ε-amino group to the γ-position does not involve exchange with free ammonia nitrogen. These data exclude an α,β-unsaturated intermediate formed by elimination of ammonia, followed by readdition of ammonia in the β-position. Also excluded would be any intermediate transfer of the amino group to a keto group (transamination) that would lead to exchange of the amino group with free ammonia. It was suggested that the reaction mechanism might involve the intermediate formation of an aziridine ring, but no evidence exists either to support or eliminate this possibility.[36]

The biosynthesis of the viomycidine fragment is not quite so clearcut. The structure of viomycidine (4) has only recently been

determined.[6,12,13] The available data indicate that viomycidine does not exist *per se* in the viomycin molecule, but exists as a precursor that gives viomycidine upon acid hydrolysis. Due to the uncertainty of the structure of the viomycidine precursor, very little direct information is available on the biosynthesis of this precursor.

Recent studies in these laboratories utilizing specifically labeled arginine precursors and one labeled ornithine precursor have demonstrated that these amino acids are incorporated into viomycin to a large extent, and that the radioactivity appears almost exclusively in viomycidine and the related guanidino compounds.[14] It is not surprising that this is true, since arginine (12), Johnson's proposed viomycidine precursor (13),[20] viomycidine (4), and the related capreomycidine (14)[13,37] are very similar in structure, as shown in Equations 2 and 3. It is also interesting to note that the viomycidine precursor (13) can be converted into capreomycidine (14) by hydrogenation of viomycin.[20]

Paśś and coworkers have investigated the origin of the guanidino group in viomycin.[38,39] They suggested that two types of enzyme systems could be possible for the introduction of the guanidino group into viomycin: 1) an arginase system responsible for catabolic breakdown of arginine to supply ureido and amidino groups, and 2) the X-amidinotransferase system that was shown by Walker to be operating in streptomycin biosynthesis.[40] It was found that although arginase activity was high when viomycin production was high, specific inhibition of the arginase resulted in only slight reduction in viomycin production, thus ruling out the possibility that the enzyme arginase is responsible for the introduction of the amidino group into viomycin.[39]



The failure of the arginase system strongly suggested that an X-amidinotransferase system was operating in viomycin biosynthesis, and this was found to be the case.[38] A strain of *Streptomyces* that produced viomycin and one that was deficient in the X-amidinotransferase system (and did not produce viomycin) were compared in a series of experiments. Both strains were found to produce the same amount of guanidino compounds, but the composition was different. Arginine concentration was found to remain constant in the producing strain, but increased sharply

with time in the nonproducing strain. Serine was found to be incorporated at the same rate in both strains, suggesting that incorporation of serine precedes that of the guanidino component.

The activity of the X-amidinotransferase system was found to be highest in the periods of intensive antibiotic production. Inhibitors such as canavanine (an antimetabolite of arginine), inorganic phosphate, and oxygen shortage were found to inhibit both the X-amidinotransferase system and viomycin production. In addition, arginine was found to be the best source of the amidino groups; *L*-α-amino-β-guanidinopropionic acid, *L*-α-amino-γ-guanidinobutyric acid, and creatine were less effective. Cyclic guanidine structures, such as creatinine, were totally ineffective as amidino group sources. The facts that the deficient strain did not produce viomycin and that arginine accumulated in the deficient strain suggested that an X-amidinotransferase system was in operation in viomycin biosynthesis and that arginine was involved in some way.

Walker has recently shown that the biosynthesis of the guanidine constituent of streptomycin, streptidine, from inosamine involves transamidination of the phosphorylated intermediate with arginine as an amidine donor. This step was believed to be followed by dephosphorylation with a nonspecific phosphatase.[40] A similar mechanism may be operative in viomycin biosynthesis. This, of course, does not rule out direct incorporation of arginine into viomycin as was indicated in previous work in this laboratory.[14]

In order to study the biosynthesis of viomycin and of the unusual

amino acid fragments of viomycin in these laboratories, viomycin was produced in the presence of ^{14}C-labeled compounds suspected of being precursors of one or more of the fragments of viomycin. The ^{14}C-labeled viomycin was then hydrolyzed using 6 *N* hydrochloric acid. The hydrolysate was then separated into individual components by use of a 2.4 cm x 450 cm column containing approximately 1650 ml of Dowex 50-WX8(H^+) ion exchange resin. Complete separation of the hydrolysis components was achieved when the column was eluted with a concentration gradient of hydrochloric acid. The individual fractions collected from this column were then analyzed for radioactivity by the liquid scintillation technique. Those hydrolysis components that were found to contain significant radioactivity were degraded further in order to determine the complete labeling patterns.[6]

Several different reactions were used to degrade the hydrolysis components to determine labeling patterns. For serine and diaminopropionic acid two separate reactions were used. Periodate oxidation followed by formation, collection, and counting of the dimedone derivative of the formaldehyde produced yielded the activity present in the C-3 position. Reaction with ninhydrin reagent by the method of van Slyke, *et al.*,[41] oxidized the carboxyl carbon (C-1) to carbon dioxide, which could then be trapped and counted to obtain the activity at C-1. The activity at C-2 was determined by difference. The β-lysine samples were degraded by use of the Barbier-Wieland degradation. The methyl ester of *N,N'*-diphthalyl-β-lysine was prepared by a modification of the method of van Tamelen.[9] The methyl ester of *N,N'*-diphthalyl-β-lysine was reacted

with phenylmagnesium bromide, and the resulting tertiary alcohol was dehydrated in boiling acetic anhydride. Ozonolysis of the resulting olefin, followed by an oxidative workup, gave very low yields of *N,N'*-diphthalylornithine and benzophenone. Ornithine was obtained by removal of the phthalyl protecting groups using hydrazine. The ornithine was then reacted with ninhydrin reagent, and the carbon dioxide that was produced was trapped in a Hyamine hydroxide solution. The activity at C-2 was determined by counting the carbon dioxide; the activity at C-1 was determined by counting the benzophenone. Activities of positions C-3 through C-6 were determined by counting the ornithine and subtracting the carbon dioxide count.

Biosynthetic studies on 14 different labeled precursors have been completed in these laboratories. Floyd investigated glucose-U-^{14}C, *DL*-serine-3-^{14}C, *DL*-serine-1-^{14}C, *DL*-lysine-2-^{14}C, *DL*-glutamic-3,4-^{14}C acid, *DL*-glutamic-5-^{14}C acid, *DL*-arginine-5-^{14}C, and *DL*-aspartic-4-^{14}C acid.[6] Rice studied sodium acetate-2-^{14}C, glycine-1-^{14}C, sodium acetate-1-^{14}C, sodium bicarbonate-^{14}C, sodium formate-^{14}C, and *DL*-lysine-1-^{14}C.[14]

Uniformly labeled glucose was chosen for the first experiment because it was expected that significant labeling would appear in each of the hydrolysis products. This precursor was used largely to test the methods of separating the hydrolysis components and counting the fractions in order to locate the radioactive ones. Except for the fact that no urea was found, every hydrolysis component was significantly labeled. Interesting data were obtained from this experiment. It was found that

those hydrolysis components that were the closest to glucose metabolically contained more radioactivity than those components that would require a large number of steps to be synthesized from glucose. The only exception was α,β-diaminopropionic acid, which contained roughly twice the activity per carbon atom that serine contained. This situation could arise if at least part of the serine to be incorporated into viomycin as a seryl residue had been bound to a cell wall prior to the addition of the labeled glucose, so that it would be available for incorporation as a serine residue, but not available for conversion to diaminopropionic acid.

In the experiments with *DL*-serine-3-^{14}C and *DL*-serine-1-^{14}C as precursors, the serine isolated from the hydrolysate contained the largest amount of activity, as might be expected. With *DL*-serine-3-^{14}C as the precursor, 42 per cent of the total activity hydrolyzed appeared in the serine fractions, while 48 per cent of the total activity was found in the serine isolated from the viomycin with *DL*-serine-1-^{14}C as the precursor. Relatively high activity was also found in the diaminopropionic acid fractions, suggesting that serine might be converted directly into diaminopropionic acid. The activity of the carbon dioxide produced in the acid hydrolysate from *DL*-serine-1-^{14}C was 5.5 times the activity of the carbon dioxide from *DL*-serine-3-^{14}C, even though the viomycin from *DL*-serine-3-^{14}C was twice as radioactive as the viomycin from *DL*-serine-1-^{14}C. This result is consistent with several of the common biological degradations serine undergoes: the conversion of a phosphatidylserine to a phosphatidylethanolamine by

decarboxylation, or conversion to pyruvate and decarboxylation of the pyruvate.[42]

The β-lysine derived from *DL*-serine-3-^{14}C also contained a significant level of activity, but very little was noted in that derived from *DL*-serine-1-^{14}C. This is consistent with at least one of the known biosynthetic pathways to lysine.[43]

Since there was evidence that β-lysine could be obtained from lysine in strains of *Clostridium* and *Escherichia*,[34,36] the next experiment, using *DL*-lysine-2-^{14}C as the precursor, was designed to determine whether lysine is the precursor of β-lysine in viomycin. A total of 41 per cent of the activity of the viomycin hydrolyzed appeared in the β-lysine fractions, clearly indicating that β-lysine is derived from lysine in viomycin biosynthesis. Both the serine and diaminopropionic acid fractions also contained relatively high activities. The ratio of the activity of the diaminopropionic acid to the activity of the serine fractions was 0.24, the same value that was obtained when *DL*-serine-1-^{14}C was used as a precursor. A direct pathway from lysine to serine would seem to be indicated by the relatively high incorporation of activity in serine when *DL*-lysine-2-^{14}C was used as a precursor.

DL-Lysine-1-^{14}C was also studied as a precursor in viomycin biosynthesis. So with the lysine-2-^{14}C experiment, the β-lysine was the most heavily labeled, while both the serine and the diaminopropionic acid were labeled significantly, but to a lesser extent than β-lysine.

The labeling patterns of the samples of serine and diaminopropionic acid that showed significant incorporation were determined in order to demonstrate more conclusively that serine is the precursor of

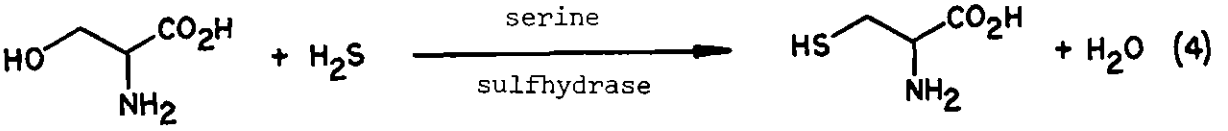
diaminopropionic acid in viomycin. The samples of serine and diaminopropionic acid from the precursors glucose-U-^{14}C, serine-1-^{14}C, serine-3-^{14}C, lysine-2-^{14}C, and lysine-1-^{14}C were subjected in the degradative reactions described earlier. The results are shown in Table 1.

Table 1. Labeling Patterns of Serine and Diaminopropionic Acid (DAPA) Derived from Labeled Viomycin as Per Cent of Specific Activity of Precursor Samples of Glucose-U-^{14}C, *DL*-Serine-1-^{14}C, *DL*-Serine-3-^{14}C, *DL*-Lysine-2-^{14}C, and *DL*-Lysine-1-^{14}C

Sample	Per Cent Activity at C-1	Per Cent Activity* at C-2	Per Cent Activity at C-3
Precursor Glucose-U-^{14}C			
Isolated Serine	32.6	33.1	34.3
Isolated DAPA	34.4	31.0	34.6
Precursor Serine-1-^{14}C	99.9	0.1	0.0
Isolated Serine	98.2	1.1	0.7
Isolated DAPA	96.3	2.7	1.0
Precursor Serine-3-^{14}C	7.8	0.4	91.8
Isolated Serine	7.8	0.3	91.9
Isolated DAPA	9.8	0.5	89.7
Precursor Lysine-2-^{14}C			
Isolated Serine	2.4	62.9	34.7
Isolated DAPA	2.1	61.0	36.9
Precursor Lysine-1-^{14}C			
Isolated Serine	98.6	1.4	0.0
Isolated DAPA	99.1	0.9	0.0

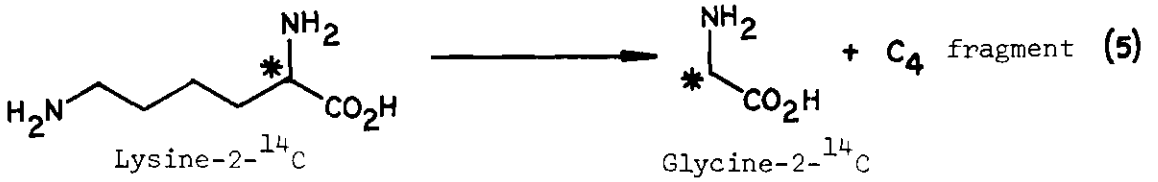
*Determined by difference: % C-2 = 100% - (% C-1 + % C-3).

Examination of the results in this table reveals that the agreement of the labeling patterns between serine and diaminopropionic acid in each case is virtually identical. These results strongly suggest that the serine unit is the direct biosynthetic precursor of the diaminopropionic acid unit. It is possible that aminomalonic acid semialdehyde is an intermediate in this conversion as was suggested by Reinbothe,[29] but other intermediates may also be possible. Another possible mechanism would involve direct addition of ammonia to serine in a manner analogous to the synthesis of cysteine from serine by addition of hydrogen sulfide in a reaction catalyzed by serine sulfhydrase, a yeast enzyme, as shown in Equation 4.[44,45]

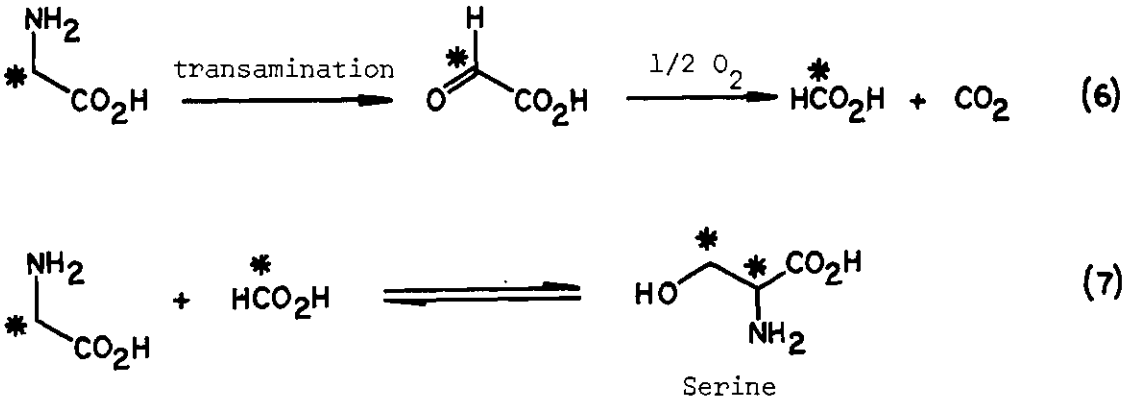


(4)

The labeling patterns of the serine and diaminopropionic acid samples from viomycin with lysine-2-^{14}C as the precursor are interesting in that the distribution of labeling was found to be two-thirds at the C-2 position and one-third at the C-3 position. A conceivable pathway is shown by Equations 5, 6, and 7.



(5)



All transformations shown except the one from lysine to glycine are known.[46] The reversible interconversion of glycine and serine is well known, and it is reasonable to expect that there would be more unlabeled formate available for reaction than unlabeled glycine. The per cent activity would then be expected to be greater at C-2 than at C-3.

The serine and diaminopropionic acid derived from lysine-1-^{14}C both had almost all of the ^{14}C activity located in the C-1 position, as can be seen from the results in Table 1. These results are completely in accordance with Equations 5, 6, and 7, as the only source of activity would be the carboxyl carbon of the glycine.

Degradation of the labeled β-lysines isolated from the viomycins derived from lysine-2-^{14}C and lysine-1-^{14}C was accomplished as described earlier. The degradation of the β-lysine from the lysine-2-^{14}C indicated that 0.8 per cent of the activity was at the C-1 position, 84.3 per cent at the C-2 position, and the remainder at the C-3 through C-6 positions. Degradation of the β-lysine from the lysine-1-^{14}C indicated that 88.3 per cent of the activity was at the C-1 position. The other positions were not counted, since these degradation reactions have low yields.

These results indicated that lysine can indeed be a direct precursor of β-lysine in viomycin biosynthesis.

DL-Arginine-5-^{14}C was investigated as a precursor, and it was found that significant activity appeared only in the viomycidine and isoviomycidine fractions and in certain fractions that follow viomycidine. These fractions apparently contain compounds more basic than viomycidine, and it would appear that these compounds are peptides which contain the viomycidine residue. One of these compounds was viocidic acid (5), which has already been described.[13,15]

Several facts support the suggestion that arginine is the precursor of the viomycidine residue in viomycin. The extremely high level of activity found in the viomycidine fractions for the arginine-5-^{14}C experiment compared to the low levels of activity found in viomycidine fractions from all other precursors investigated is significant. The next most active viomycidine sample was derived from the glucose-U-^{14}C experiment, which contained less than 3 per cent of the activity of the viomycidine from arginine-5-^{14}C. It is interesting to note that arginine and viomycidine are both guanidino amino acids, and that their molecular formulas differ by only four hydrogen atoms.

Each of the precursors *DL*-glutamic-3,4-^{14}C acid, *DL*-glutamic-5-^{14}C acid, *DL*-aspartic-4-^{14}C acid, sodium acetate-2-^{14}C, sodium acetate-1-^{14}C, glycine-1-^{14}C, sodium bicarbonate-^{14}C, and sodium formate-^{14}C gave very low incorporation of activity into viomycin, and none of the hydrolysis products showed significant activity.

The purposes of this research were twofold: first, to continue the investigations into the biosynthetic pathways leading to viomycin by

studying several suspected biological precursors of the amino acids and other components in viomycin; and second, to further investigate the chemistry of the intact viomycin molecule, derivatives of viomycin, and their hydrolysis products.

CHAPTER II

EXPERIMENTAL

Apparatus and Techniques

Spectra

Nuclear magnetic resonance spectra (60 MHz) were obtained using Varian Associates Models A-60A and A-60D spectrometers equipped with a variable temperature attachment. Chemical shift values are reported in τ units ($\tau = 10 - \delta$). Internal standards used were tetramethylsilane (TMS) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

All low resolution mass spectral data were obtained using a Varian Associates Model M-66 mass spectrometer. Exact mass determinations were obtained by linear interpolation between ions of an internal standard of known mass. Intensity values of individual peaks were determined by averaging the intensities obtained from several scans.

Infrared spectra were obtained using Perkin-Elmer Models 457 and 137 spectrophotometers. The spectra of solids were obtained from potassium bromide pellets pressed at 2500 psig.

Ultraviolet spectra were obtained using a Cary Model 14 spectrophotometer. Matched 1.0 cm quartz cells were used. The spectra were recorded as solutions in distilled water, 0.1 *N* hydrochloric acid, or 0.1 *N* sodium hydroxide solution, except where otherwise noted.

Chromatography

Thin layer chromatography was used for qualitative analysis, and the plates were prepared as described previously.[47] Ninhydrin spray reagent was used for visualization of spots corresponding to amino acids.[48] Weber's reagent, as previously described, was used for visualization of spots corresponding to guanidine and guanidino compounds;[48] *p*-dimethylaminobenzaldehyde reagent was used to visualize spots corresponding to urea.[48] Ultraviolet lamps (254 nm and 366 nm filters) were also used for visualization of spots on tlc plates coated with silica gel HF_{254} (Brinkman Instruments, Inc.).

Paper chromatography was used for both qualitative analysis and preparative separations. Chromatographic papers Whatman Number 1 and Whatman Number 3MM were used for qualitative analysis, and Whatman Number 17 was used for preparative chromatography. All paper chromatograms were developed by the descending solvent technique in a glass tank that was fully equilibrated between solvent and vapor.

A Beckman Model R electrophoresis apparatus, Beckman number 320046, was used for electrophoretic separations. All analyses were done using Beckman number 319328 paper strips and Beckman number 319329 paper wicks. All electrophoresis experiments were done at a constant voltage. The voltage, distance of migration from the origin, direction of migration, buffer used, and the time of the current flow are presented with each experiment. Detection of the bands on the strips was accomplished in the same manner as for thin layer and paper chromatography.

Gel filtration chromatography was used for purification and

separation purposes using Sephadex G-15 (Pharmacia Fine Chemical, Inc.) prepared and used as described previously.[47]

Ion exchange resins were used for the conversion of peptide salts into their free base forms, exchange of the anions in peptide salts, and for the separation of amino acids and peptides in hydrolysates. The resins used were regenerated and used as described previously.[6,10] The following abbreviations are used: IR-45(OH^-), IR-45($SO_4^=$), and IR-45(Cl^-) for Amberlite anion exchange resin 45 in the hydroxyl, sulfate, and chloride phases, respectively; IRC-50(H^+) for Amberlite cation exchange resin 50 in the hydrogen phase; and Dowex 50(H^+) for Dowex 50W X-8 (100-200 mesh, Baker reagent 1930) in the hydrogen phase.

Miscellaneous

Optical rotations were determined using a Bellingham and Stanley Model No. 397619 polarimeter with the sodium D line as a light source.

Unless otherwise stated, all concentrations and evaporations were performed using a modified, all glass Rinco (Model VE-1000A) rotary evaporator at water aspirator vacuum and at temperatures not in excess of 60°C.

All melting points were obtained using a Köfler hot stage and are corrected. Microanalyses were performed by Bernhardt Laboratories (Mülheim, West Germany). All bioassays were conducted by Dr. Paul D. Shaw of the University of Illinois, Urbana, Illinois.

A Welsbach Model T-408 ozone generator was used in all ozonolyses. Ozone was generated at 90 volts with a flow rate of 0.5 standard liters per minute (s.l.p.m.) and a pressure of 8 psig.

A GM Instrument Company, Inc. Model VE-2002-B-24 automatic fraction collector was used to collect timed fractions from chromatography columns.

All catalytic hydrogenations were performed at atmospheric pressure and room temperature; the data were corrected to standard conditions. The catalyst was a 10 per cent platinum on carbon mixture (Engelhard Industries, Inc., Lot No. C-3246) and was used in a 50 per cent aqueous acetic acid solvent. The catalyst-solvent mixture was equilibrated with hydrogen for 12 hr or more before introduction of the sample.

Techniques for Liquid Scintillation Counting

All counting of radioactive samples was done using a Packard Tri-Carb Model 3375 Liquid Scintillation Spectrometer. All samples were counted using the green channel (with the blue channel as a check) with the C-D channel selector in (C:050, D:1000). Since no radioactive isotopes other than ^{14}C were present, these discriminator settings gave the widest possible energy "window" for counting, thus improving the counting efficiency. The print-out was normally set to print AES ratio, sample number, time, gross counts for the green channel, and net counts per minute (cpm) for the green channel. Occasionally, the blue channel was also printed out to cross-check the results obtained on the green channel. The following dial settings were normally made:

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

Background counts were determined using a standard series of samples made up as close in composition as possible to the samples to be counted, but without any radioactivity. These background samples were counted several times for 500 min each using the same dial settings that would be used for radioactive samples, except that the background subtraction was set to zero. The average value of the background counts was set on the background subtraction dial, and the counter was then ready for use.

A toluene-Triton X-100 scintillation solution (tT21) was used for all water-soluble samples.[49] This scintillation solution was prepared by dissolving 6.936 g of 2,5-diphenyloxazole (PPO) and 0.173 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in two liters of distilled toluene. After solution was complete, 1 kg of Triton X-100 (Reg. Trademark of Rohm & Haas, Inc.) purified for liquid scintillation counting (Packard Instrument Company, Inc., Cat. No. 6008083) was added with stirring. The scintillation solution was then ready for use. Normal background counts for this solution were 20 to 30 cpm.

The scintillation samples for the determination of ^{14}C activity were made up by diluting 250 µl aliquots of the desired sample with 750 µl of water (dispensed with a continuous flow Cornwall pipettor) in a counting vial. Fourteen milliliters of tT21 scintillation solution was then added to the vial (dispensed with a 20 ml LabIndustries L/I Repipet, Cat. No. 3020-A, set to deliver 14.0 ml of the solution). The vial was then capped, shaken, and allowed to stand until a clear solution resulted. After being placed in the freezer compartment of the

scintillation spectrometer, all samples were allowed to equilibrate for at least 30 min before being counted.

Counting efficiencies were determined by a method different from that described by Floyd.[6] Due to difficulties with the automatic external standardization (AES) system on the scintillation spectrometer, the AES ratios were reliable only over short ranges and were not generally used to correlate quenching with counting efficiency. Instead, a standard series of samples containing exactly known amounts of benzoic-^{14}C acid were made up using water and hydrochloric acid concentrations ranging from 0.1 *N* to 6.0 *N*. These samples were made up in exactly the same manner as the routine counting samples. The per cent counting efficiency was calculated for each sample in the standard series, and these counting efficiencies were plotted on a graph versus acid concentration (normality) in each sample. Since Floyd has determined the normality of the hydrochloric acid required to elute each of the hydrolysis components of viomycin from the long Dowex 50(H^+) column,[6] a counting efficiency could be determined for each component that is eluted from the column. This method has proven to be far more accurate than any other method previously used.

The total activity (dpm) of each fraction was determined as shown in Equation 8.

$$(\text{dpm}) = \frac{(\text{cpm}/250\ \mu\text{l sample})(4)(\text{no. milliliters in fraction})}{(\text{counting efficiency})} \qquad (8)$$

Fractions containing significant amounts of the same hydrolysate component were pooled, evaporated to dryness, redissolved in 15 ml of water, and lyophilized. These samples were saved for purification, counting, and degradation studies to determine the complete ^{14}C-labeling patterns.

Chemical Studies

The Catalytic Hydrogenation of Viomycin

Several samples of commercial viomycin sulfate were catalytically hydrogenated. In a typical reduction, a quantity of commercial viomycin sulfate was placed in a desiccator and dried over Drierite (Hammond Drierite Co.) under vacuum. This dried material still contained approximately 10 per cent water, as well as some inorganic salts that act as buffers in aqueous solution.

A 6.783 g sample of this material was dissolved in 50 ml of 50 per cent aqueous acetic acid. In the meantime, 7.5 g of 10 per cent platinum on carbon catalyst was slurried with 250 ml of 50 per cent aqueous acetic acid and equilibrated with hydrogen at room temperature and atmospheric pressure. After this time, the viomycin sample was introduced with careful exclusion of air. This mixture was then stirred for 410 hr, during which time 520 ml of hydrogen (STP) was absorbed by the viomycin and the uptake of hydrogen had virtually stopped. The catalyst was removed by filtration through a bed of Celite, and the filtrate was evaporated to a colorless glass. This material was then dissolved in 50 ml of water and lyophilized to give 7.286 g of a white, amorphous solid, which was named perhydroviomycin. This material was stirred with

90 ml of IR-45(OH^-) resin until the *p*H was 5.5. The resin was then filtered and washed with water; the filtrate and the washings were combined and evaporated to a thin syrup. This was applied to and eluted through a column containing 60 ml of IR-45($SO_4^=$) with 250 ml of water. Lyophilization of the eluate gave 6.240 g of perhydroviomycin as the stoichiometric sulfate salt.

The uv spectra of this material in aqueous solution (*p*H 5), in 0.1 *N* hydrochloric acid, and in 0.1 *N* sodium hydroxide showed no absorption. The ir spectrum was similar in general appearance to that of viomycin.

The nmr spectrum of perhydroviomycin in deuterium oxide (13 per cent, w/v) was very similar to the spectrum of viomycin, except that the prominent absorption at τ 1.93 in the spectrum of viomycin had completely disappeared.

Tlc analyses of perhydroviomycin using water, 1-butanol:acetic acid:water:acetone:3 *N* ammonium hydroxide (9:2:4:3:2), and 1-butanol: acetic acid:water (4:5:1) each revealed only one ninhydrin positive spot, which had a different R_f value from viomycin in the first two systems, but a similar R_f value in the third system. Nessler's and *p*-dimethylaminobenzaldehyde reagents revealed no ammonium ion or urea present in the perhydroviomycin preparation.

The biological activity of this material was measured by the zone inhibition method using *B. subtilis* and was found to be approximately 30 per cent as active as viomycin.

As a further check of the purity of perhydroviomycin, 2.371 g of

perhydroviomycin sulfate was subjected to gel filtration chromatography over a 550 cm x 1.9 cm Sephadex G-15 column. Approximately 1400 ml of wet Sephadex was slurried in one liter of 0.01 *N* formic acid, poured into the column, and allowed to settle by gravity for two days. The perhydroviomycin was dissolved in 10 ml of 0.01 *N* formic acid and was introduced onto the column. The column was eluted with 1800 ml of 0.01 *N* formic acid, and 15 ml fractions were collected. The fractions were individually lyophilized, and a weight curve was plotted (Figure 1). A total

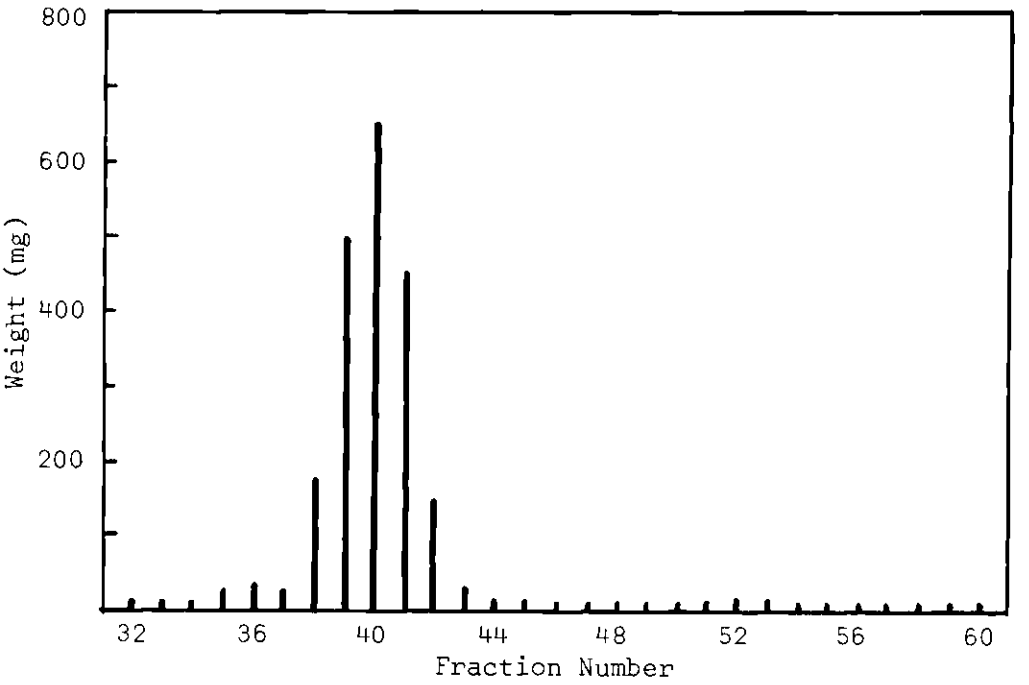


Figure 1. Weight Curve for the Sephadex Chromatography of Perhydroviomycin

of 2.190 g of material (92.4 per cent) was recovered, of which 93 per cent was homogeneous, giving a Gaussian peak in the weight curve. Bioassay of each fraction in the peak revealed an average activity of

30 per cent of the activity of commercial viomycin. The uv spectra of each of the fractions in water showed no absorption except end absorption ($E^{1\%}_{1\ cm}$ less than 7).

Hydrolysis of Perhydroviomycin

Perhydroviomycin samples were hydrolyzed in much the same manner as previous samples of viomycin,[6] except that the carbon dioxide produced was collected for weight determination. In a typical experiment, 2.890 g of perhydroviomycin was hydrolyzed using 6 *N* hydrochloric acid and a nitrogen purge.[6] A *t*-butyl alcohol trap, a cold trap, and a tube containing magnesium perchlorate were added to the hydrolysis apparatus to remove hydrogen chloride, hydrochloric acid, *t*-butyl chloride, and water vapor from the gas stream. A tube containing Ascarite (Arthur H. Thomas Co.) and a small amount of magnesium perchlorate was used to trap the carbon dioxide. The increase in weight of this tube was taken as the net carbon dioxide produced. A background weight was determined by bubbling nitrogen through a hydrolysis mixture that contained only 6 *N* hydrochloric acid, under the usual conditions. Known samples of viomycin were hydrolyzed under the same conditions; approximately 75 per cent of the theoretical quantity of carbon dioxide was trapped. The carbon dioxide trapped from the perhydroviomycin hydrolysate was 74.8 per cent of the theoretical amount expected.

The hydrolysis mixture was removed from the steam bath and was evaporated to a thin syrup. Excess hydrochloric acid was neutralized to *p*H 4 with IR-45(OH^-) resin. The resin was then filtered and washed well with water. The filtrate and washings were combined and

concentrated to a volume of approximately 5 ml. This was chromatographed over the long Dowex 50(H^+) column previously described by Floyd.[6] Four hundred fractions were collected and were tested with ninhydrin, Nessler's, and Weber's reagents to detect amino acids, ammonium ion, and guanidino compounds, respectively. The fractions collected from this column and the identities of the hydrolysate components are shown in Table 2. Each of the ninhydrin positive fractions was lyophilized and the weights were recorded. The total weight of each component is also given in Table 2.

The acid hydrolysate of perhydroviomycin was subjected to tlc analysis in the solvent systems previously mentioned, with the additional system: *t*-butyl alcohol:acetic acid:water:5% ammonium hydroxide (5:4:1:3). Serine, alanine, diaminopropionic acid, and β-lysine were positively identified in this way by direct comparison with known compounds and by comparison of colors given with the ninhydrin reagent. Nessler's reagent revealed the presence of ammonium ion in the alanine fractions, but no urea was detected in any fraction or on tlc analysis by use of *p*-dimethylaminobenzaldehyde reagent.

Viomycidine and the related capreomycidine[37] were found to give rather different colors with both Weber's reagent and ninhydrin reagent. Samples of known viomycidine and capreomycidine were compared with the unidentified guanidino-containing material from the acid hydrolysate of perhydroviomycin. Both the ninhydrin and Weber tests of the unidentified material closely matched the colors given by capreomycidine on tlc plates. Tlc analysis of this material, viomycidine, and capreomycidine

Table 2. Hydrolysis Components of Perhydroviomycin

Fractions	Identity	Weight (mg)	Millimoles[a]	Ratio[a]
142-157	Serine	477(746)[a]	3.38	2.00
185-198	Alanine	160(249)[b]	1.80	1.06
188-197	Ammonium Chloride	99[b]	1.88	1.11
265-276	Diaminopropionic Acid	304(482)[a]	2.17	1.28
282-298	β-Lysine	650	c	(1)
303-321	Capreomycidine	320	c	(1)

[a]Weights in parentheses are weights of the lyophilized fractions; Floyd[6] determined that serine and diaminopropionic acid both gain about 36 per cent in weight upon lyophilization from 4.0 *N* hydrochloric acid. The weights and number of millimoles shown have been corrected for this.

[b]Alanine and ammonium ion overlap on the column; the weight shown for ammonium chloride is the weight lost from the alanine fractions upon removal of ammonia.

[c]No weight gain data are available for β-lysine and capreomycidine; the ratio shown is inferred by comparison of the weights obtained from this experiment with those from other experiments.

in the usual solvent systems revealed that the guanidino material from perhydroviomycin was identical with capreomycidine.

No other major components were found in the acid hydrolysate of perhydroviomycin. Except for the absence of urea, these results are in agreement with those found by Johnson and coworkers.[13,20]

In order to obtain an accurate weight for the alanine, it was necessary to remove the ammonium chloride which was found in most of the alanine-containing fractions. All of the alanine-containing fractions were combined, lyophilized, and stored under vacuum to remove all traces of excess hydrochloric acid. The dry powder (348 mg) was slurried with 20 ml of dry IR-45(OH^-) resin and 50 ml of absolute ethanol and evaporated to dryness. This procedure was repeated four times. After the last evaporation, 50 ml of 0.2 *N* hydrochloric acid was added to dissolve the alanine. A Nessler's test of this solution was negative, showing that the ammonium ion had been removed. The resin was filtered and washed with 50 ml of 0.2 *N* hydrochloric acid. The filtrate and washings were combined and lyophilized to dryness. A total of 249 mg of alanine was isolated. The loss in weight of 99 mg was assumed to be the weight of ammonium chloride that was removed. A weight gain of 36 per cent was assumed for the alanine, based on the behavior of serine, in the mole ratio calculations. A small amount of this alanine was recrystallized from ethanol-water and was used for an optical rotation. The alanine was found to be optically inactive ($[\alpha]_D^{22}$ 0° (±2°), *c*, 0.75 in water, *c*, 0.44 in glacial acetic acid; lit. values for *L*-alanine: $[\alpha]_D^{25}$ + 1.8°, *c*, 2 in water, $[\alpha]_D^{25}$ + 33.0°, *c*, 2 in glacial acid[50]).

Biosynthetic Studies

Preparation and Purification of Labeled Viomycin Samples

Samples of viomycin investigated in this study were produced from an isolate of *Streptomyces griseus* var. *purpurea* at the University of Illinois under the direction of Dr. Paul D. Shaw. Production of viomycin was achieved as previously described.[6] Each batch of viomycin produced was assayed by uv spectrophotometry, biologically by the zone inhibition method using *B. subtilis*, and by thin layer chromatography in the solvent system: *t*-butyl alcohol:acetone:acetic acid:5 per cent ammonium hydroxide:water (5:5:5:3:1).

Seven radioactive compounds and three compounds labeled with ^{15}N and ^{2}H suspected of being precursors of viomycin were added to the culture medium in separate experiments, and the viomycin that was produced was isolated and purified. The precursors studied and their respective run numbers are listed in Table 3. This table also summarizes the data obtained by private communication from Dr. Paul D. Shaw concerning the samples of viomycin produced using these compounds as precursors.[51]

Hydrolysis and Chromatographic Techniques for Labeled Viomycin Samples

Hydrolyses of radioactively labeled viomycin were routinely carried out as follows: the sample of radioactively labeled viomycin was weighed and diluted with one to two grams of commercial viomycin (Parke, Davis, and Company) that had been dried to constant weight in a vacuum desiccator at room temperature. This sample was then dissolved in 80 ml of 6 *N* hydrochloric acid, placed under a reflux condenser, and heated at steam bath temperature for 20 hr. Nitrogen was slowly bubbled through

Table 3. The Production of Labeled Viomycin

Run No.	Precursor	Precursor Added	Viomycin Isolated Weight (mg)	^{14}C Activity (dpm)	Per Cent Incorporation
15	*DL*-Arginine-amidine-^{14}C	4.93×10^{8} dpm	168	9.22×10^{6}	5.40
16	*DL*-Ornithine-1-^{14}C	6.86×10^{8} dpm	200	1.72×10^{7}	2.32
17	*DL*-Arginine-1-^{14}C	7.06×10^{8} dpm	156	1.28×10^{7}	2.34
18	*D*-Glucose-U-^{14}C	2.16×10^{10} dpm	1061	1.55×10^{8}	0.78
19	*L*-α,β-Diaminopropionic-U-^{14}C Acid	2.09×10^{7} dpm	186	6.55×10^{5}	3.74
20	*L*-β-Lysine-^{14}C (labeling pattern undetermined)	1.43×10^{7} dpm	157	1.07×10^{6}	8.15
21	*L*-Viomycidine-^{14}C (labeling pattern undetermined)	6.92×10^{6} dpm	319	7.58×10^{4}	1.09
22	*L*-Lysine-^{15}N	0.296 mmoles	71	-	3.54[a]
23	*L*-Serine-^{15}N	0.438 mmoles	142	-	2.26[a]
24	3,3-Dideuterioserine	0.762 mmoles	114	-	1.38[b]

[a]Average values based on previous experiments using ^{14}C-labeled precursors.

[b]Based on an experiment using serine-3-^{14}C (Run No. II, Floyd[6]).

the hydrolysis solution and into a trap containing approximately ten milliliters of a one molar solution of the hydroxide of Hyamine 10-X (Reg. Trademark of Rohm & Haas, Inc.) in methanol that was connected to the reflux condenser. The carbon dioxide released during hydrolysis was trapped as the carbonate salt of Hyamine hydroxide.[52] For counting, the methanolic solution of Hyamine hydroxide was diluted to 50.00 ml with methanol. A 250 µl aliquot was used for counting and was treated as already described.

After the hydrolysis was completed, the hydrolysate was concentrated to a syrup. Excess hydrochloric acid was removed under vacuum. The syrup was then dissolved in 20 ml of water and IR-45(OH^-) resin was added until the *p*H was about five. The solution was filtered, and the resin was washed with four to six 20 ml portions of water. The filtrate and washings were then combined and concentrated to a volume of 2 to 5 ml; the sample was then ready to be chromatographed.

The column used to chromatograph the viomycin hydrolysate was the same one used by Floyd.[6] The Dowex 50(H^+) resin was used as described previously,[6] except that only 200 ml of water was added to the wet resin. This allowed the entire column, which had a volume of 1750 ml, to be filled in one pouring, resulting in a more evenly distributed column. The resin was allowed to settle by gravity for at least four hours; the stopcock was then opened, and the excess water was drained. The column was then ready for use.

The hydrolysate sample was pipetted onto the top of the column

and washed onto the resin with a small amount of water. The column was then eluted by gradient elution chromatography with hydrochloric acid as described previously.[6]

The automatic fraction collector (GM Instrument Company, Inc. Model VE-2002-B24) was modified to a capacity of 234 18 × 150 mm test tubes. Fractions of approximately 15 ml each were collected using the automatic timer function set for 15 min fractions. The column flow rate was adjusted periodically to 12 drops per minute in order to maintain 15 ml fractions. The modifications were necessary in order to increase the reliability of the fraction collecting system.

Although the column was designed to separate the hydrolysate of five grams of viomycin, samples as small as 100 mg were chromatographed with excellent results.

The quantities of labeled and unlabeled viomycin hydrolyzed in these experiments are listed in Table 4.

A summary of the total activities found in each of the principal fractions from the hydrolysates of the radioactively labeled viomycins is shown in Table 5.

Plots of radioactivities of the fractions obtained from the chromatography of the hydrolysates of these viomycin samples are shown in Figures 2 through 8.

As can be seen from Figures 2-8 and from Table 5, the guanidino compounds were significantly labeled when arginine-amidine-^{14}C, ornithine-1-^{14}C, and arginine-1-^{14}C were used as precursors. The other components of the hydrolysate showed very little incorporation from these precursors.

Table 4. Amounts of Viomycin Hydrolyzed for Each Experiment

Run No.	Precursor	Labeled Viomycin* (mg)	Unlabeled Viomycin Added* (mg)	Total (mg)
15	Arginine-amidine-^{14}C	168	2459	2627
16	Ornithine-1-^{14}C	200	2541	2741
17	Arginine-1-^{14}C	156	2233	2389
18	Glucose-U-^{14}C	1004	0	1004
19	Diaminopropionic-U-^{14}C Acid	182	1459	1641
20	β-Lysine-^{14}C (labeling pattern undetermined)	157	1624	1781
21	Viomycidine-^{14}C (labeling pattern undetermined)	319	1028	1347
22	Lysine-^{15}N	156	0	156
23	Serine-^{15}N	226	0	226
24	3,3-Dideuterioserine	170	0	170

*Expressed as milligrams of the sulfate salt dried to constant weight at 25°C.

Due to the unavailability of ^{14}C-labeled diaminopropionic acid, β-lysine, and viomycidine, the glucose-U-^{14}C experiment (Run No. 1)[6] was repeated using a larger quantity of more highly labeled glucose (Run No. 18). This preparation was hydrolyzed without the addition of unlabeled viomycin. The diaminopropionic acid, β-lysine, and viomycidine components were isolated and separately used as precursors for

Table 5. Summary of the Principal Fractions from the Hydrolysate of Radioactively Labeled Viomycin

Precursor	Fractions	Identity	Weight (mg)	Total Activity (dpm)
Arginine-amidine-^{14}C (Run No. 15)				
	132-142	Serine	784	12,000
	209-227	-	128	286,700
	240-251	DAPA	304	121,800
	253-268	β-Lysine	664	89,500
	269-284	Isoviomycidine	123	999,300
	285-304	Viomycidine	283	2,276,000
	305-325	Peptides	40	320,000
	326-358	Peptides	15	816,900
	359-400	Peptides	253	1,269,000
	-	Carbon dioxide	-	87,000
Ornithine-1-^{14}C (Run No. 16)				
	165-177	Serine	450	111,000
	200-208	-	43	32,000
	260-272	-	267	109,500
	292-312	DAPA	221	366,000
	314-332	β-Lysine	777	97,500
	333-349	Isoviomycidine	131	907,000
	350-365	Viomycidine	361	3,740,000
	366-400	Peptides	81	477,000
	401-420	Peptides	74	245,000
	421-446	Peptides	183	1,532,000
	-	Carbon dioxide	-	182,000
Arginine-1-^{14}C (Run No. 17)				
	191-205	Serine	871	151,200
	324-335	DAPA	444	66,100
	343-355	β-Lysine	641	48,000
	360-387	Isoviomycidine	202	1,275,000
	388-405	Viomycidine	320	2,742,000
	411-425	Peptides	61	433,500
	435-451	Peptides	42	305,000
	-	Carbon dioxide	-	182,000

Table 5. Summary of the Principal Fractions from the Hydrolysate of Radioactively Labeled Viomycin (Continued)

Precursor	Fractions	Identity	Weight (mg)	Total Activity (dpm)
Glucose-U-^{14}C (Run No. 18)				
	93-101	-	-*	254,000
	155-168	Serine	202	40,000,000
	169-297	-	-*	9,380,000
	300-310	DAPA	141	24,600,000
	311-316	-	-*	1,010,000
	317-329	β-Lysine	175	15,100,000
	330-345	Isoviomycidine	-*	2,590,000
	346-370	Viomycidine	92	7,470,000
	371-390	Peptides	-*	2,070,000
	391-420	Peptides	-*	133,000
	421-454	Peptides	-*	6,860,000
	-	Carbon Dioxide	-	12,700,000
L-α,β-Diaminopropionic-U-^{14}C Acid (Run No. 19)				
	144-154	Serine	-*	22,000
	278-289	DAPA	280	233,000
	-	Carbon Dioxide	-	116,000
L-β-Lysine-^{14}C (Labeling pattern undetermined) (Run No. 20)				
	392-410	β-Lysine	417	1,030,000
	-	Carbon dioxide	-	5,500
L-Viomycidine-^{14}C (Labeling Pattern undetermined) (Run No. 21)	No significant activity in any fraction.			

*Not isolated

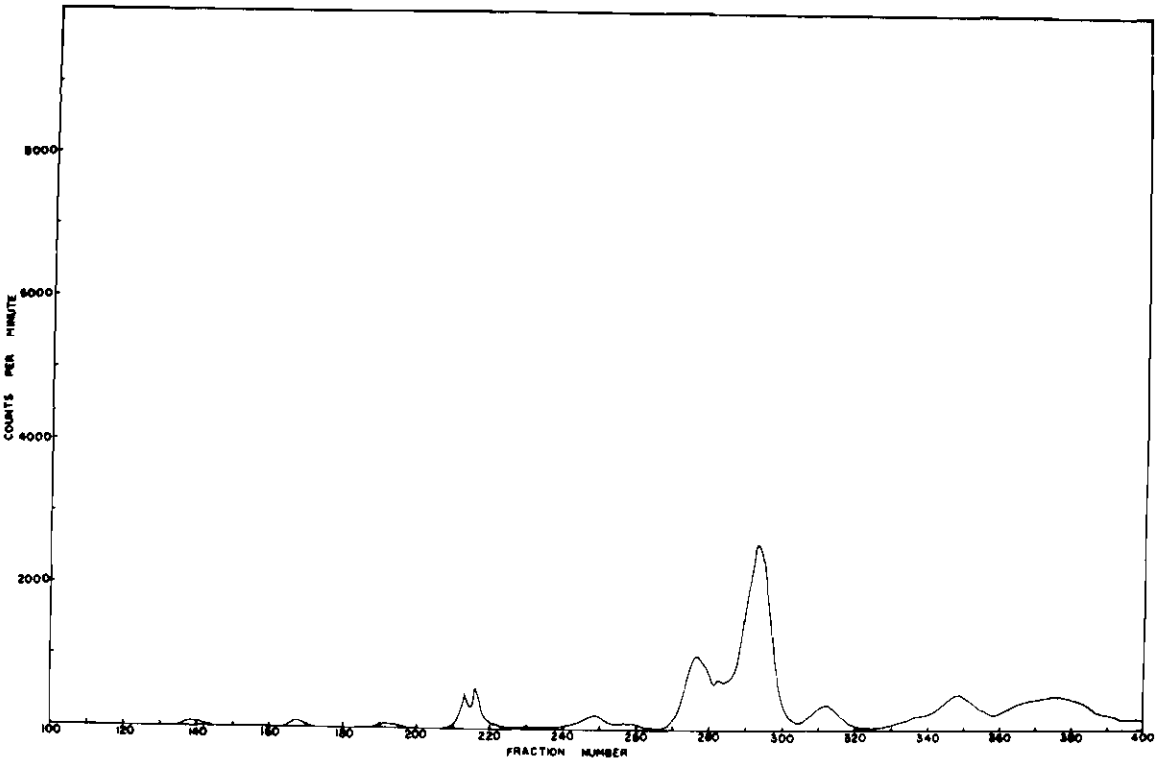


Figure 2. Activity Curve for the Hydrolysate of Viomycin from *DL*-Arginine-Amidine-^{14}C (Run No. 15)

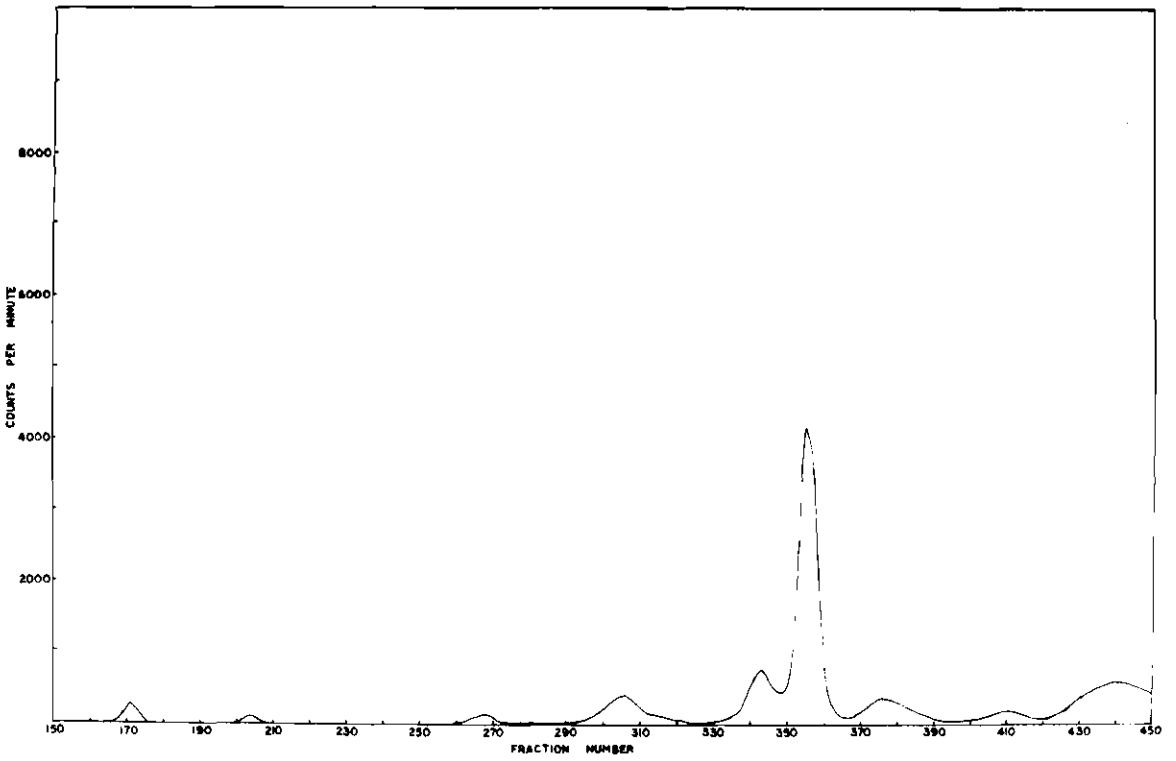


Figure 3. Activity Curve for the Hydrolysate of Viomycin from *DL*-Ornithine-1-^{14}C (Run No. 16)

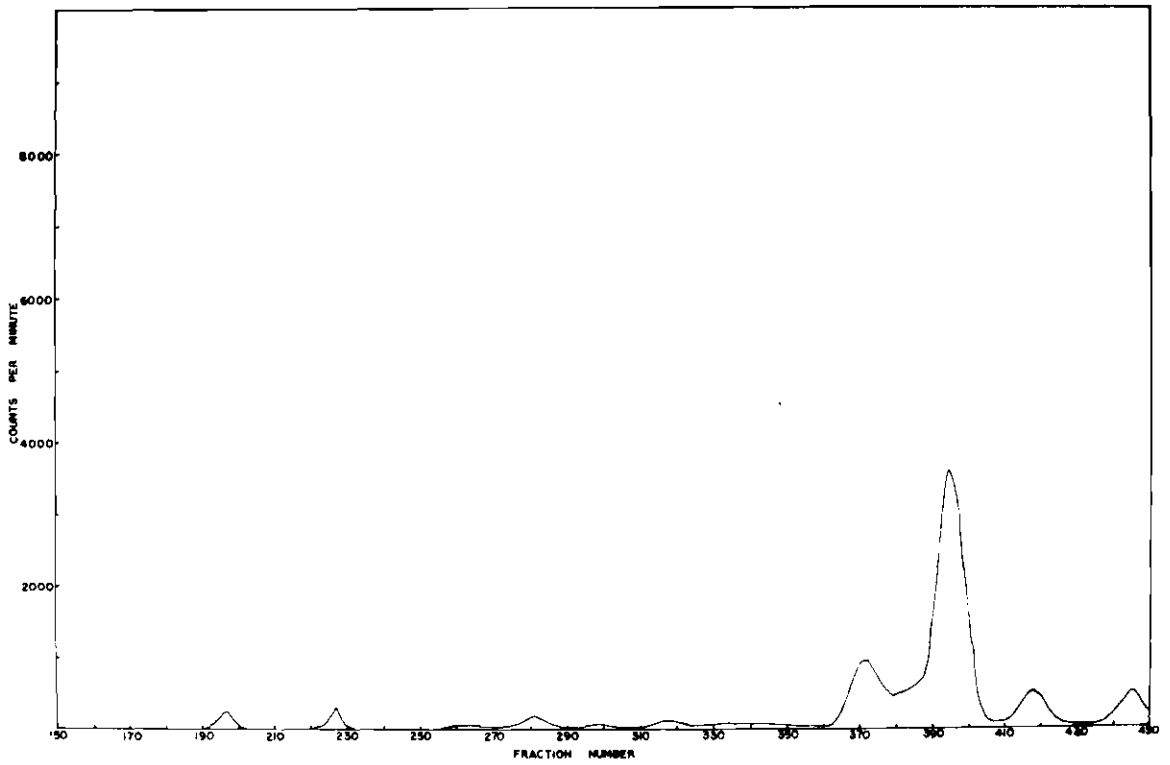


Figure 4. Activity Curve for the Hydrolysate of Viomycin from *DL*-Arginine-1-^{14}C (Run No. 17)

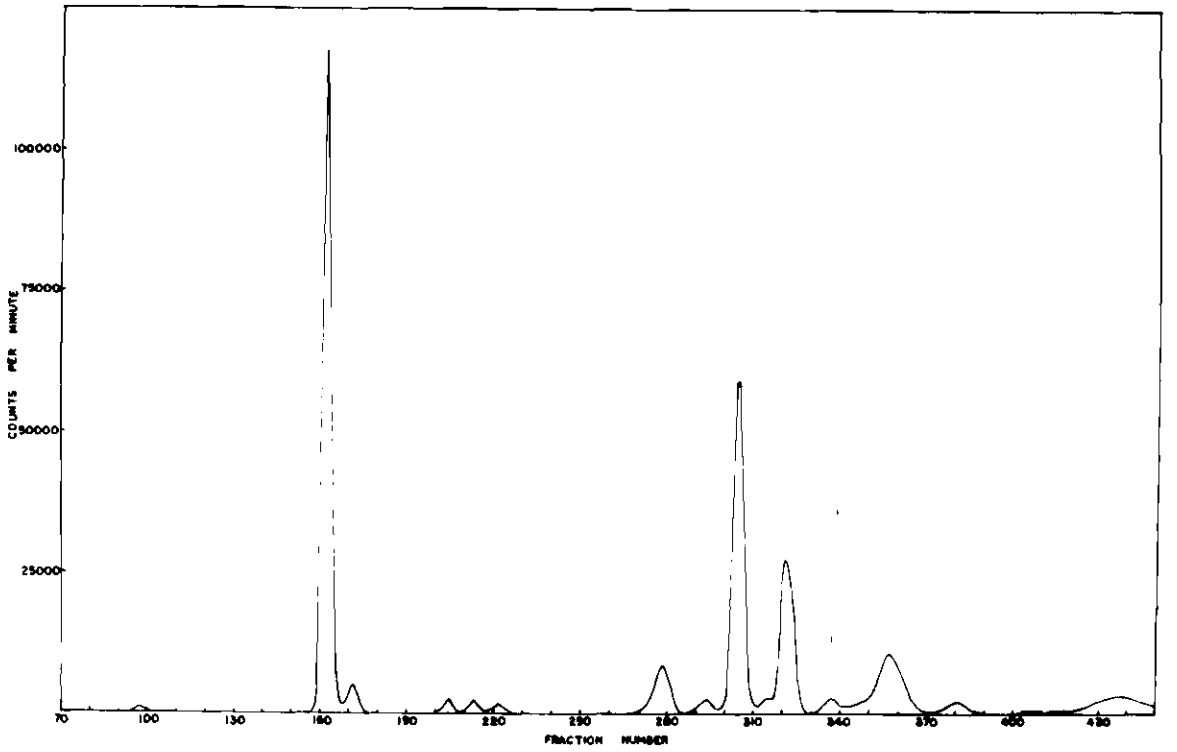


Figure 5. Activity Curve for the Hydrolysate of Viomycin from *DL*-Glucose-U-^{14}C (Run No. 18)

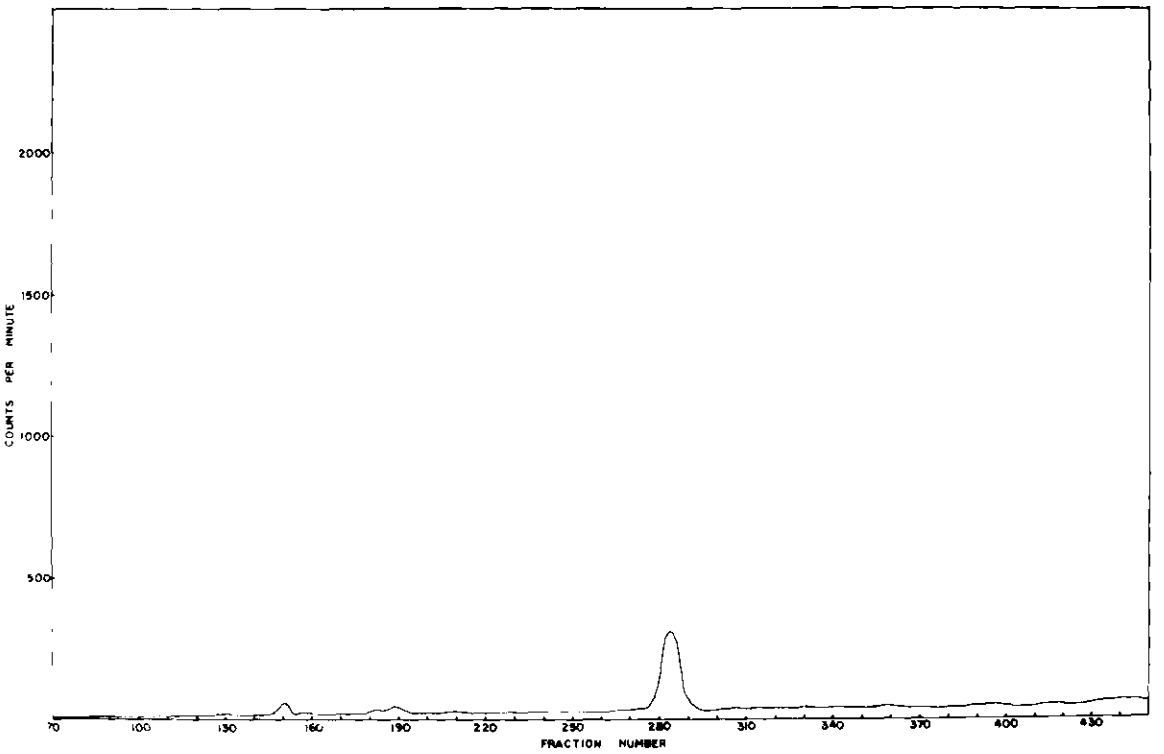


Figure 6. Activity Curve for the Hydrolysate of Viomycin for *L*-α,β-Diaminopropionic-U-^{14}C Acid (Run No. 19)

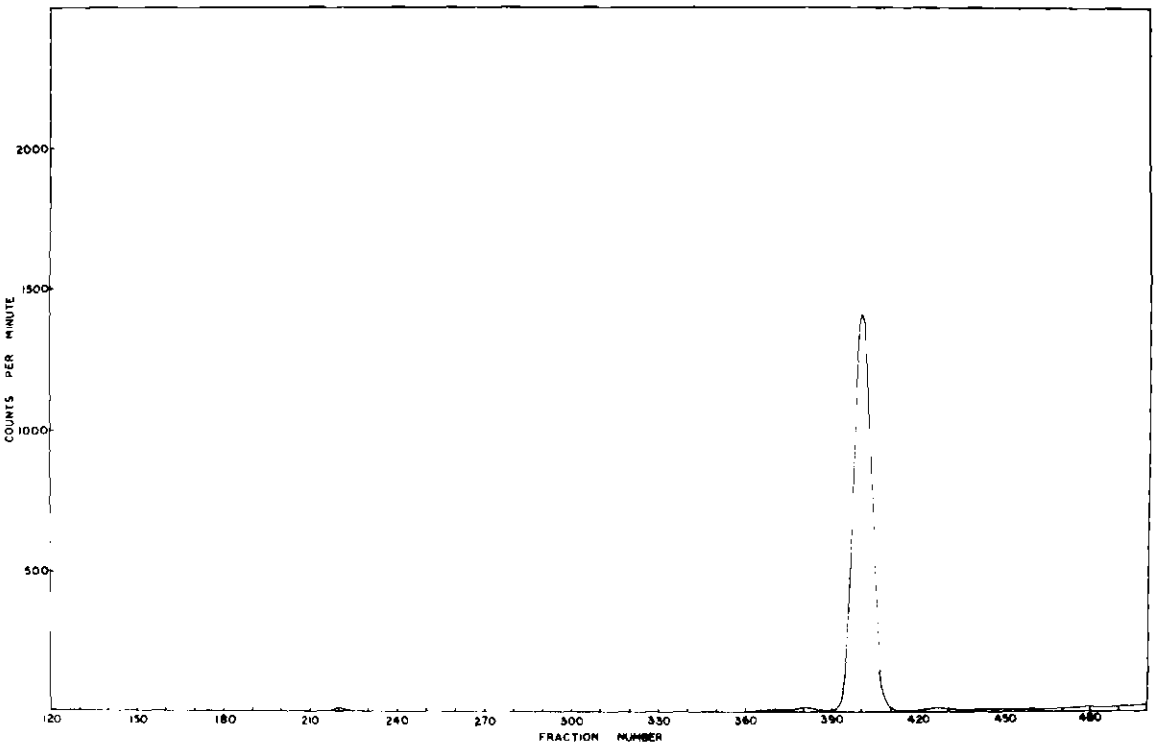


Figure 7. Activity Curve for the Hydrolysate of Viomycin from *L*-β-Lysine-^{14}C (Run No. 20)

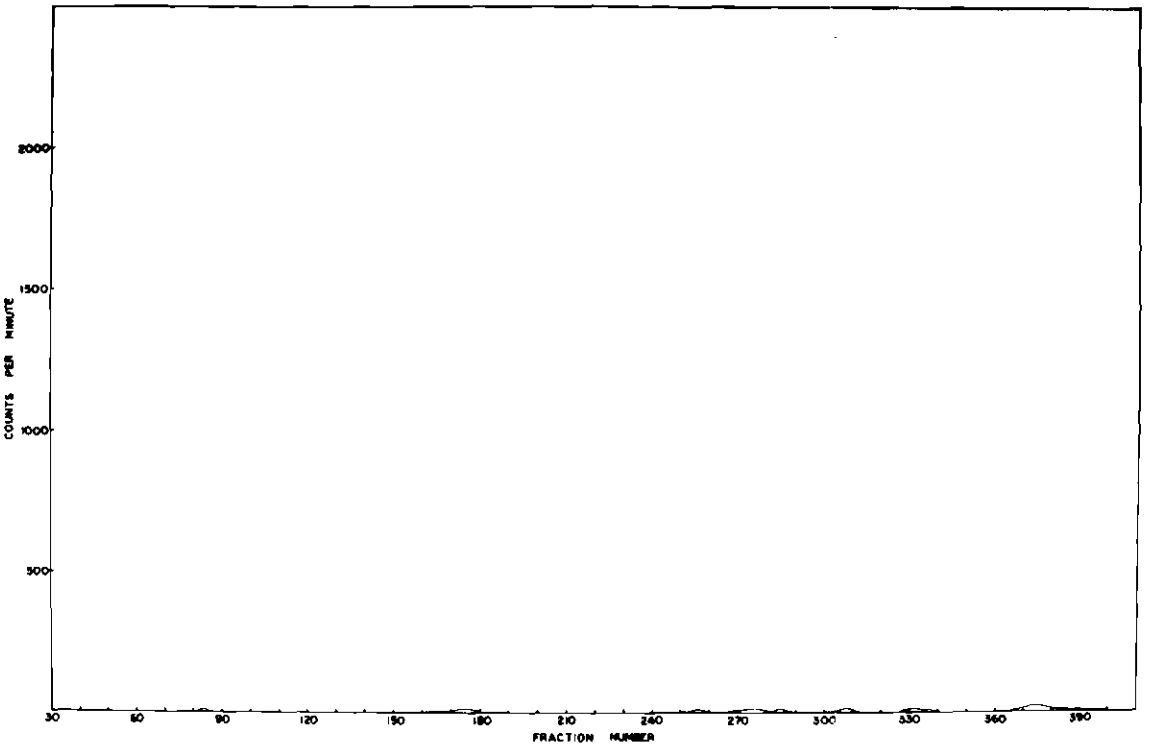


Figure 8. Activity Curve for the Hydrolysate of Viomycin from *L*-Viomycidine-^{14}C (Run No. 21)

incorporation into viomycin. These viomycin samples were then hydrolyzed, chromatographed, and counted (Run Nos. 19, 20, and 21).

Determination of the Specific Activities of the Hydrolysis Components from ^{14}C-Labeled Viomycin Samples

Diaminopropionic Acid. The lyophilized sample of diaminopropionic acid isolated from Run No. 19 was crystallized as described by Floyd.[6] A 49.9 mg sample of the crystalline diaminopropionic acid monohydrochloride was dissolved in water and diluted to 5.00 ml. A drop of chloroform was added to prevent the growth of mold. A 100 µl aliquot was placed in a counting vial, and 900 µl of water and 14.0 ml of tT2l scintillation solution were added. The activity was counted as usual and the counting efficiency was determined by comparison of the AES ratio with those of solutions of known *p*H. The specific activity

was calculated from Equation (9).

$$S.A. = \frac{(cpm)(10)}{(E)(c)} \qquad (9)$$

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

cpm = activity of sample

E = counting efficiency of sample

c = concentration of sample in mg/ml.

The results are given in Table 6. The remainder of the diaminopropionic acid solution was saved for subsequent degradation studies.

Viomycidine. Due to the difficulties involved in crystallizing viomycidine, the viomycidine samples (isolated from Run Nos. 7, 15, 16, and 17) were not crystallized. The specific activities were determined in another way. Each of the viomycidine samples was dissolved in 15 ml of water and lyophilized to dryness. The samples were then stored under vacuum in a desiccator over Linde 4A molecular seive pellets (Union Carbide Corporation) for four days. The samples were weighed accurately, and 10.0 ml of water was added to each one. After each sample was dissolved, 1.00 ml of each one was diluted to 10.00 ml in a volumetric flask. A 250 µl aliquot of each sample was placed in a counting vial, and 750 µl of water and 14.0 ml of tT21 scintillation solution were added. The samples were counted as usual, and the specific activities were calculated from Equation 10. Counting efficiencies were determined in the same manner as for the diaminopropionic acid.

$$S.A. = \frac{(cpm)}{(E)(w)} \qquad (10)$$

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

cpm = activity of sample

E = counting efficiency of sample

w = weight of sample counted.

The weight counted, w, was determined by assuming that the total weight of the viomycidine sample was accurately diluted 1:400. The 250 µl aliquot actually counted thus represented 1/400 of the total sample weight. The results are given in Table 6. The unused portions of each viomycidine sample were combined with the original samples and relyophilized for degradation studies.

Determination of the ^{14}C-Labeling Patterns of the Hydrolysis Components from Viomycin Samples

Diaminopropionic Acid: The C-3 Carbon. The solutions of each sample of diaminopropionic acid used in the determination of the specific activities were also used in the degradation reactions. For selectively determining the activity at the C-3 position, a periodate oxidation was used. For each sample, 2.00 ml of the solution of the amino acid was added to a 25 ml Erlenmeyer flask containing 4.5 ml of 0.5 *M* acetic acid-sodium acetate buffer, *p*H 4.7. To this solution was added 2.4 ml of a freshly prepared solution of 0.5 *M* sodium *meta*-periodate (107 mg/ml), and the mixture was allowed to stand at room temperature for two and one-half hours. After this time, 2.00 ml of an ethanolic solution of dimedone (80 mg/ml of ethanol) was added,

and the dimedone derivative of the formaldehyde produced by the periodate oxidation was allowed to crystallize for 20 hr. The crystalline precipitate was then filtered, washed with water, and recrystallized from ethanol-water. The recrystallized derivative was filtered, washed with water, and dried. In calculations, one milligram of the dimedone derivative of formaldehyde is equivalent to 0.4746 mg of diaminopropionic acid monohydrochloride.

The radioactive dimedone derivatives were counted by weighing to the nearest tenth of a milligram approximately ten milligrams of the crystalline derivative into a counting vial. Fifteen milliliters of tT2l scintillation solution was added, and the samples were counted as usual. The results of these degradations are given in Table 6.

Diaminopropionic Acid: The C-1 Carbon. Although previous degradations of serine and diaminopropionic acid used both *N*-bromosuccinimide oxidation and ninhydrin oxidation to determine the activity at C-1,[6,14] only the ninhydrin oxidation was found to give accurate and reliable results from diaminopropionic acid.[6] The samples of diaminopropionic acid were reacted with ninhydrin by a modification of the manometric method of van Slyke and coworkers,[41] as described by Floyd.[6] A 50 µl aliquot of the diaminopropionic acid solution was added to 3 ml of a 1 *M* citric acid-sodium citrate buffer, *p*H 2.5, in the reaction compartment of the reaction flask. Fifty milligrams of ninhydrin was added to this solution with a spatula; then an empty vial was put into place in the receptacle, and the flask was tightly stoppered with a rubber serum cap. The reaction flask was allowed to stand

in a boiling water bath for ten minutes and was then removed and cooled to room temperature. As soon as the reaction mixture was cool, 0.5 ml of the 1 *M* solution of the hydroxide of Hyamine in methanol was injected into the vial and the reaction flask was shaken on a rotary shaker for three hours. The small vial was then removed and placed *in toto* in a scintillation vial, and 14.0 ml of tT21 scintillation solution was added. The vial was allowed to stand in the freezer compartment of the scintillation spectrometer for three days in total darkness before accurate counts were obtained. Counting efficiency was determined by adding 0.50 ml of a solution of benzoic-^{14}C acid in toluene (917 dpm/ml) and comparing the observed count rate with the known disintegration rate. The results of this degradation are given in Table 6.

The activity at the C-2 position was determined by taking the difference between the total activity and the sum of the activities at C-1 and C-3.

Viomycidine. Nassar[16] has found previously that viomycidine was degraded into guanidine and *DL*-aspartic acid by acetylation, ozonolysis of the acetylated viomycidine (hydrogen peroxide workup), and acid hydrolysis. Thus the amidine carbon (C-6)* was determined directly from

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*The standard method of numbering the bicyclic ring system of viomycidine does not lend itself to a clear description of the relationships of labeling patterns between the precursors and viomycidine. Therefore, the numbering system used in these degradations will refer to the standard numbering system for the arginine and ornithine precursors used. The structures and numbering system used for ornithine, arginine,

the guanidine produced, and the carboxyl carbon (C-1) was determined by ninhydrin oxidation of the aspartic acid. The C-5 carbon is apparently lost as carbon dioxide during the ozonolysis and subsequent workup, and attempts were made to trap and count it directly. The total activity of the other carbon atoms of aspartic acid was determined both by the difference between the activity of the aspartic acid and the activity of the carbon dioxide produced in the ninhydrin oxidation of aspartic acid, and by direct counting of an aliquot of the residual ninhydrin oxidation reaction mixture.

To the lyophilized samples of viomycidine derived from the precursors arginine-amidine-^{14}C, ornithine-1-^{14}C, and arginine-1-^{14}C, 30 ml of dry pyridine and 30 ml of acetic anhydride were added, and the solution was heated on a steam bath for 20 hr. A drying tube filled with Drierite was used to exclude water vapor from the reaction mixture. After this time, the solution was cooled to room temperature, and 60 ml of water was cautiously added. The solution was allowed to stand at room temperature for 2 hr, and then the dark brown liquid was evaporated

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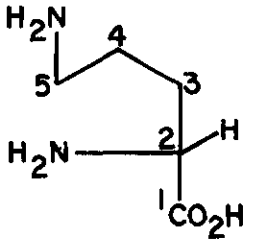
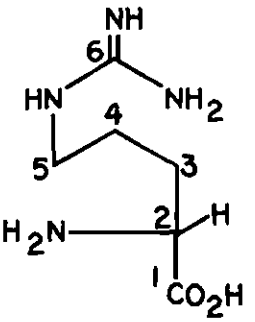
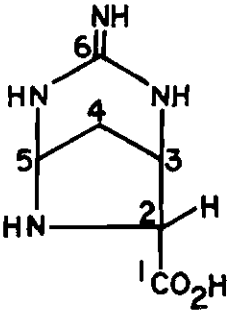
and viomycidine are shown in structural formulas 15, 12, and 4, respectively.



15
ornithine



12
arginine



4
viomycidine

to a thin syrup. Another 40 ml of water was added and the solution was again evaporated to a syrup.

This syrup was dissolved in 75 ml of 80 per cent formic acid and ozone was bubbled through the solution at 0°C for 1.5 hr. After this time, 5.0 ml of 30 per cent hydrogen peroxide was added and the solution was allowed to stand overnight. Another 5.0 ml of the hydrogen peroxide solution was added to the orange solution, and the resulting solution was allowed to stand for one hour. The solution was then evaporated to a syrup, and 75 ml of 6 *N* hydrochloric acid was added. This solution was heated on a steam bath for 22 hr and was then removed from the steam bath and stirred with a teaspoon of Darco G-60 for 4 to 6 hr. The carbon was then filtered through a bed of Celite, and the pale yellow filtrate was evaporated to a syrupy crystalline mass.[16] This material was dissolved in 5.0 ml of water, and the guanidine, aspartic acid, and other components were separated by preparative paper chromatography. On a 17 cm × 46 cm Whatman No. 17 paper strip was placed 0.75 ml of this solution, and the paper strip was dried thoroughly. The paper was then chromatographed using 95 per cent ethanol:3 *N* ammonium hydroxide (5:1) in a descending chromatography apparatus that was in equilibrium with the solvent vapor. After the solvent had traveled the entire length of the strip, the papergram was removed from the tank and dried in air for several hours. A small test strip was sprayed with Weber's reagent and ninhydrin reagent to locate the guanidine and aspartic acid bands, respectively. Typically, the guanidine had an R_f value of 0.65, while the aspartic acid had an R_f value of 0.15. The test strips were used as

a guide for cutting the bands from the paper strips. Only the center portions of the bands were used, since guanidine and an unidentified band tended to overlap at their edges.

The guanidine-containing strips and the aspartic acid-containing strips were cut into small pieces and separately extracted with three 30 ml portions of water. The aqueous extracts were separately filtered and then lyophilized. The samples were then weighed, and 3.00 ml of water was added to each one. A 250 µl aliquot was taken for counting as usual. Counting efficiencies were determined by adding 250 µl of the 917 dpm/ml solution of benzoic-^{14}C acid in toluene used previously to the sample vials after counting the activity already present, as previously described. The results of these degradations are shown in Table 6. The remainder of each aspartic acid solution was saved for further degradation.

The viomycidine sample derived from arginine-5-^{14}C was treated in a slightly different manner. The acetylation reaction was carried out as described for the other viomycidine samples. Since it was expected that the C-5 carbon would be lost during ozonolysis and subsequent workup, the ozonolysis reaction, hydrogen peroxide workup, and acid hydrolysis steps were arranged such that any carbon dioxide produced would be trapped. The ozone was passed through the sample solution, bubbled through a trap containing styrene to remove all traces of ozone, and then into a trap containing Hyamine hydroxide. After the ozonolysis, 5.0 ml of 30 per cent hydrogen peroxide was added to the ozonolysis reaction mixture using a syringe through a rubber septum. The reaction

mixture was allowed to stand overnight at room temperature. An additional 5.0 ml of 30 per cent hydrogen peroxide was added using the syringe, and a slow purge of oxygen was bubbled through the solution and the traps for 2 hr. The ozonized solution was evaporated to a syrup, and 75 ml of 6 *N* hydrochloric acid was added. The solution was heated on a steam bath for 22 hr. A slow nitrogen purge was bubbled through the hydrolysate and into a trap containing Hyamine hydroxide. The Hyamine hydroxide solutions from the ozonolysis and the acid hydrolysis traps were separately diluted to 50.0 ml with methanol and counted as usual. The total activity of carbon dioxide produced is given in Table 6.

The acid hydrolysate was treated as previously described and the results derived from it are also shown in Table 6.

The aspartic acid isolated from each of the degradations of the viomycidine samples derived from arginine-1-^{14}C and ornithine-1-^{14}C was subjected to further degradation by the ninhydrin oxidation reaction. Duplicate determinations were carried out as described for diaminopropionic acid. Samples of 100 µl and 250 µl of each of the aspartic acid solutions were used. The Hyamine hydroxide was counted as described for the diaminopropionic acid to determine the ^{14}C activity at C-1. The sum of the activities at C-2, 3, and 4 was determined by difference between the activity of the aspartic acid and that of the carbon dioxide released during the oxidation. The reaction solution was also counted as a check. The results are given in Table 6.

Table 6. Specific Activities and Labeling Patterns of Diaminopropionic Acid and Viomycidine Derived from Radioactively Labeled Viomycin

Precursor	Compound	Specific Activity (dpm/mg)[a]	Carbon[b] Number	Activity[c] (dpm)	Per Cent of Total Activity Per Atom
Diaminopropionic-U ^{14}C Acid (Run No. 19)					
	DAPA	981.0 (138.2)	C-1	271	27.6
			C-2	390	39.2
			C-3	320	33.2
Arginine-amidine-^{14}C (Run No. 15)					
	Viomycidine	8237 (1681)	C-1, C-2,3,4	140	7.9
			C-5	-[d]	-
			C-6	1820	92.1
Ornithine-1-^{14}C (Run No. 16)	Viomycidine	11608 (2369)	C-1	1794	48.6
			C-2,3,4	1788	48.4
			C-5	-[d]	-
			C-6	113	3.1
Arginine-1-^{14}C (Run No. 17)	Viomycidine	8166 (1666)	C-1	1575	91.4
			C-2,3,4	59	3.4
			C-5	-[d]	-
			C-6	89	5.2
Arginine-5-^{14}C (Run No. 7)					
	Viomycidine	20220 (4126)	C-1, C-2,3,4	170	<1.0
			C-5	2.04×10^5	100[e]
			C-6	13	<1.0

[a]The number in parentheses is the specific activity in dpm per μmole.

[b]These numbers are assigned as explained in the note on p. 54, this thesis.

[c]The activity is given in dpm per 250 μl sample of the standard solutions of the various materials degraded as described in the text.

[d]Activity not determined and assumed to be 0 per cent.

[e]Determined by difference.

Since the viomycidine from the ornithine-1-^{14}C experiment was not labeled predominately at C-1, a sample of the *DL*-ornithine-1-^{14}C used as the precursor was degraded in order to determine whether the ornithine was in fact specifically labeled at C-1 or was more randomly labeled.

Approximately one microcurie of the *DL*-ornithine-1-^{14}C that was used as the precursor for Run No. 16 was dissolved in water, and 47.5 mg of unlabeled *L*-ornithine monohydrochloride was added. This solution was diluted to exactly 5.00 ml, and a 250 µl aliquot was counted. Aliquots of this solution were subjected to ninhydrin oxidation by the same method used for the diaminopropionic acid and aspartic acid samples. The Hyamine hydroxide solutions used to trap the carbon dioxide produced were dissolved separately in 14.0 ml of the tT21 scintillation solution and counted. A 250 µl aliquot from each of the residual ninhydrin reaction solutions was dissolved in 0.75 ml of water and 14.0 ml of the scintillation solution and counted. An average of 98.2 per cent of the ^{14}C activity was found at C-1 of the ornithine.

Viomycin from ^{15}N- and Deuterium-Labeled Precursors

In addition to the ^{14}C-labeling experiments, *L*-lysine-^{15}N (Run No. 22), *L*-serine-^{15}N (Run No. 23), and 3,3-dideuterioserine (Run No. 24) were also used as precursors. The production data are shown in Table 3, and the hydrolysis data are shown in Table 4. The viomycin sample from each precursor was hydrolyzed and chromatographed using the same techniques as for the ^{14}C-labeled viomycin samples. Each of the hydrolysis components was isolated, and the serine, diaminopropionic acid, and β-lysine were subjected to low resolution mass spectral analysis.[14] The results of this study are given in Table 7.

Table 7. Low Resolution Mass Spectral Studies of the Hydrolysis Components of Viomycin from ^{15}N- and Deuterium-Labeled Precursors

Hydrolysis Product	Precursor for Viomycin Biosynthesis	Per Cent Incorporation[a]	MS Voltage	m/e Examined	Indicated Atom Excess (Per Cent)
Serine (Run No. 22)	Lysine-^{15}N	0.19	30	60/61	1.66 (^{15}N)
DAPA·HCl (Run No. 22)	Lysine-^{15}N	0.04	30	30/31 59/60	0.85 at C-3 1.82 at C-2 + C-3 0.97 at C-2[c]
β-Lysine (Run No. 22)	Lysine-^{15}N	0.57	70	56/57 101/102/103	1.86 at ε-NH_2 3.97 at m/e 102 (^{15}N) 2.70 at m/e 103 ($2^{15}N$)
Serine (Run No. 23)	Serine-^{15}N	1.00[b]	30	60/61	0.91 (^{15}N)
DAPA·HCl (Run No. 23)	Serine-^{15}N	0.22	30	30/31 59/60	2.92 at C-3 (^{15}N) 4.75 at C-2 + C-3 1.83 at C-2[c]
β-Lysine (Run No. 23)	Serine-^{15}N	0.05	70	56/57 101/102/103	0.04 at ε-NH_2 0.80 at m/e 102 (^{15}N) 2.32 at m/e 103 ($2^{15}N$)
Serine (Run No. 24)	3,3-Dideuterioserine	0.59	30	60/61/62	1.32 at M+1 (D) 0.65 at M+2 (D_2)
DAPA·HCl (Run No. 24)	3,3-Dideuterioserine	0.14	30	59/60/61	6.15 at M+1 (D) 1.78 at M+2 (D_2)

[a]Based on the level of incorporation obtained in the appropriate ^{14}C-labeling experiments.

[b]Average of results from serine-1-^{14}C and serine-3-^{14}C incorporation experiments.

[c]Determined by difference.

CHAPTER III

DISCUSSION OF RESULTS

The purpose of this research was to continue the study of the biosynthesis of the antibiotic viomycin by *Streptomyces griseus* var. *purpurea*. Viomycin is a peptide that yields upon acid hydrolysis the three unusual amino acids, *L*-α,β-diaminopropionic acid, *L*-β-lysine, and a guanidino amino acid named viomycidine, in addition to the more common compounds *L*-serine, ammonia, and carbon dioxide. The major area of investigation of this research was the study of the biosynthesis of each of these unusual amino acids, with special attention to the viomycidine component. Also investigated during this research was some further aspects of the chemical behavior of viomycin.

The Chemistry of Viomycin

Information presented in the recent literature[13,20] suggested that catalytic hydrogenation of viomycin be studied. It was reported that catalytic hydrogenation of viomycin under undefined conditions gave urea and a perhydro derivative of viomycin that possessed no uv chromophore. Upon acid hydrolysis of this perhydro derivative, *L*-serine, *L*-α,β-diaminopropionic acid, *L*-β-lysine, capreomycidine, and alanine were produced in a molar ratio of 2:1:1:1:1. The alanine isolated was found to be optically active, and this optical activity was attributed to stereospecific hydrogenation. Since the method for determining the molar

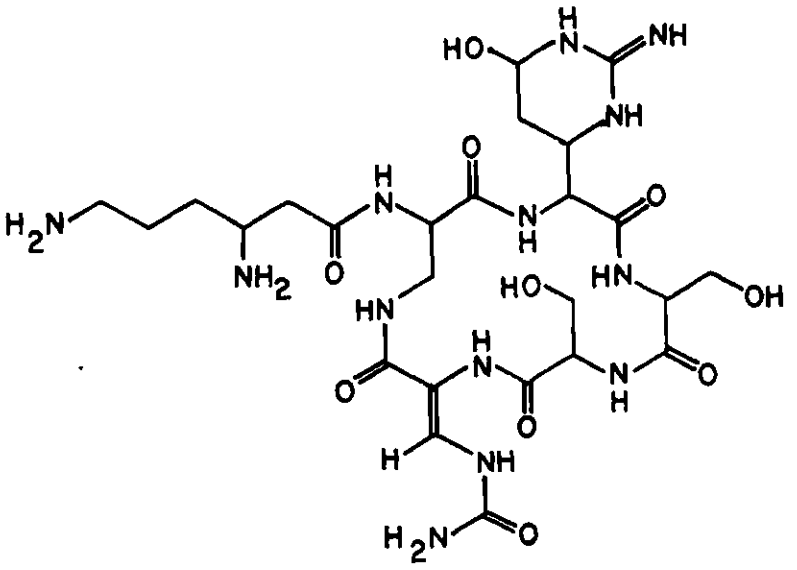
ratio was not reported, it seemed possible that the ratio might not be accurate. It also seemed unlikely that optically active alanine would be produced under the hydrogenation conditions used, and it therefore seemed advisable to repeat this experiment.

It was found that viomycin was catalytically hydrogenated in 50 per cent aqueous acetic acid solvent using a 10 per cent platinum on carbon catalyst at room temperature and atmospheric pressure. For a five to seven gram sample of viomycin sulfate, the time required for complete hydrogenation was usually 400 hr or more.

From three separate hydrogenation experiments, an average of 2.64 mmole of hydrogen (STP) was absorbed per millimole of commercial viomycin sulfate. As mentioned previously, however, commercial viomycin sulfate contains some inorganic salts, which serve as buffering agents in aqueous solution, as well as some water of hydration that is not completely removed under the conditions used to dry the viomycin sample prior to hydrogenation. If 10 per cent is deducted from the weight of each viomycin sample to account for these impurities, an average of 2.94 mmole of hydrogen (STP) was absorbed per millimole of viomycin sulfate.

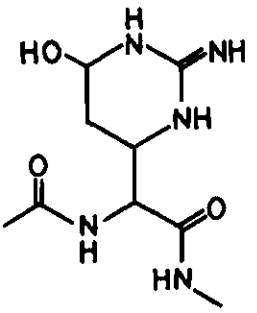
During the course of the biosynthetic studies of viomycin, 25 different samples of viomycin were produced using the usual commercial preparation procedure. An average purity of 84.3 per cent was obtained for these samples of viomycin sulfate, as determined by the analytical methods mentioned previously.[21] Thus, an allowance of 10 per cent for impurities is not unreasonable.

Although the reaction sites of the three equivalents of hydrogen are not known with any certainty, the experimental results may be at least partially explained using the structure of viomycin (9) proposed by Johnson.[13]

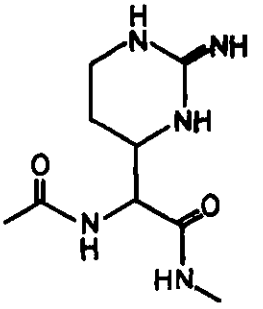


9

One equivalent of the hydrogen was proposed to be consumed in a hydrogenolysis of the hydroxyl group in the viomycidine precursor (shown as partial structure 9a) to give partial structure 9b. Acid hydrolysis of 9b would give the related compound capreomycidine (14), which was reported as a hydrolysis product of perhydroviomycin.[20]

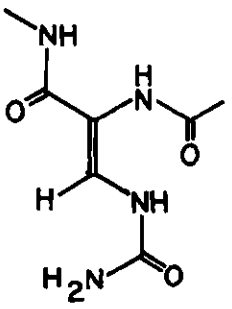


9a

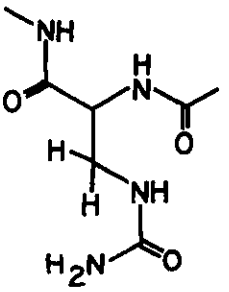


9b

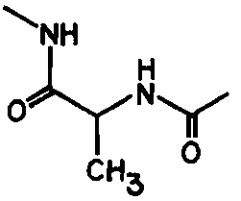
A second equivalent of hydrogen would be consumed by reduction of the olefin in structure 9 (as shown by partial structure 9c) to give partial structure 9d, while the third equivalent of hydrogen was



9c

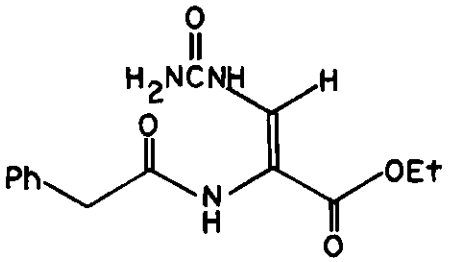


9d



9e

proposed to be consumed in the hydrogenolytic cleavage of urea from 9d to give 9e. Upon acid hydrolysis, 9e would give alanine. Johnson supported this proposal by synthesizing a model compound (16) that was reported to give alanine in good yield upon catalytic hydrogenation, followed by acid hydrolysis. Oxidation with potassium permanganate was reported to give formylurea.[53] Similar results had previously been reported by Johnson for viomycin and capreomycin.[20]



16

The alanine isolated was reported to be optically active ($[\alpha]_D^{24}$ - 4.7°, *c*, 0.3 in water; lit. value for *D*-alanine, $[\alpha]_D^{25}$ - 1.8°, *c*, 2 in water[50]), and the optical activity was attributed to a stereospecific hydrogenation.

Since the method of determining the molar ratio of the hydrolysis products of perhydroviomycin was not reported by Johnson, some doubt existed as to the accuracy of his results. It is possible that the alanine produced might be the result of a hydrogenolysis of the C-3 hydroxyl of a serine residue. Thus, the serine:alanine ratio would be expected to be more nearly 1:1. In view of these possible discrepancies, it seemed advisable to repeat Johnson's work.

Somewhat different results were obtained in attempts to repeat this work. The product of hydrogenation of viomycin sulfate was shown to be a ninhydrin positive material that had no ultraviolet chromophore. Sephadex chromatography of the crude hydrogenation product showed that it was 93 per cent homogeneous. Nessler's and *p*-dimethylaminobenzaldehyde tests revealed that no ammonium ion or urea was present in the crude hydrogenation product. Acid hydrolysis of this material gave serine, alanine, ammonium chloride, diaminopropionic acid, β-lysine,

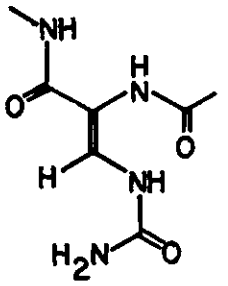
and capreomycidine (but no urea), as well as some minor peptides. The serine to alanine ratio was found to be 2:1.

The fact that no urea was found in the crude perhydroviomycin preparation or in the acid hydrolysate of perhydroviomycin is further evidence that "old viomycin" and "new viomycin" are in fact different antibiotics chemically. Simple removal of the carbamoyl group from structure 9 would result in a vinylamine system, which is known to be hydrolyzed very rapidly in aqueous solution.[54] Thus, "new" and "old" viomycin may differ by more than just a urea residue.

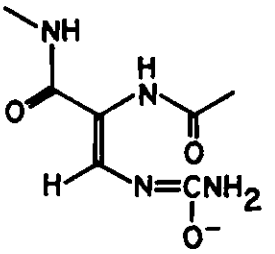
The serine to alanine ratio demonstrates with reasonable certainty that alanine is not derived from serine by hydrogenolysis of the C-3 hydroxyl of serine. Since alanine is not found in the acid hydrolysate of viomycin, it is possible that the alanine is derived from the chromophore in viomycin or from a nonchromophoric pre-alanine unit by reduction with hydrogen. Under the hydrogenation conditions used, however, it seems unlikely that such a reaction would be stereospecific and result in optically active alanine, as reported by Johnson.[20] Optically inactive alanine was isolated from three separate experiments that were designed to repeat Johnson's work. This result is more consistent with the usual nonstereospecific course followed by most hydrogenations. This fact also confirms that the alanine is not derived from serine by hydrogenolysis, since alanine produced in this manner would be expected to be optically active.

Several other inconsistencies in Johnson's structure for viomycin (9) are apparent. He suggests that 9c is responsible for the uv

chromophore of viomycin, and that the shift in the uv in alkaline solution is due to a "phenol" shift of the chromophore to give a resonance stabilized anion such as 9f. To support this proposal, Johnson



9c

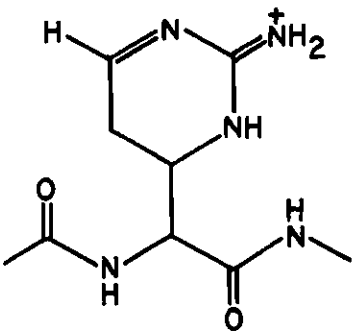


9f

synthesized a model compound (16) and reported that this compound undergoes such a shift in the uv upon addition of alkali.[53] This compound was also reported to have a pK_a value of 12.6 in water; this was cited as evidence that the model closely resembles viomycin, which has pK_a values of 8.3, 10.3, and 12.2 in water.

Johnson, however, does not take into account the greater basicity of the guanidine group in the viomycidine precursor 9a, which would more likely be responsible for the pK_a value of 12.2 in viomycin. In addition, the fact that both "old viomycin" (which contains a urea residue) and "new viomycin" (which does not contain urea) have the same pK_a values, as well as the same uv absorption, indicates that Johnson's structure for viomycin (9) is not correct.

Perhaps a better structure for the uv chromophoric center would be as shown in part structure 17. This structure could be expected to yield viomycidine (4) under conditions of acid hydrolysis. Catalytic



17

hydrogenation, followed by acid hydrolysis, would also be expected to give capreomycidine, as observed experimentally. Although Johnson reported that his model compound (16) has a one proton singlet at τ 2.2 in the nmr spectrum (hexadeuteriodimethylsulfoxide-deuterium oxide solvent),[53] the olefinic proton of 17 that is adjacent to the guanidino function is in a very electronegative environment and would be expected to be strongly deshielded and thus would appear at very low field. The guanidino group could easily account for the pK_a value of 12.2 in viomycin, and the extended system of electron density would be expected to absorb strongly in the uv, which should shift in alkaline solution due to deprotonization of the guanidino function.

In view of the many inconsistencies of Johnson's structure with the available data, it is clear that structure 9 is incorrect. The data at hand, however, are not sufficient to allow the proposal of a more suitable structure.

It is interesting to note that although hydrogenation of viomycin destroys the uv chromophore completely, biological activity is retained to the extent of 30 per cent by perhydroviomycin. In all other cases of

chemical alteration of viomycin, destruction of the uv chromophore was accompanied by loss of biological activity. These facts and a complete description of perhydroviomycin and the related perhydrocapreomycin have been reported to the offices of scientific research at the Georgia Institute of Technology and the National Institutes of Health. Record of Invention and Disclosure of Invention forms have also been filed.[55]

The Biosynthetic Studies of Viomycin

The proposed method of investigation of the biosynthesis of viomycin was to produce viomycin in the presence of ^{14}C-labeled compounds suspected of being precursors of one or more of the fragments of viomycin. The methods used for production of viomycin were similar in every respect to those used in earlier biosynthetic studies of viomycin.[6,14,21] The ^{14}C-labeled viomycin was hydrolyzed; the individual hydrolysis components were separated, and the activity of each component was determined as previously described.[6] Further degradation was planned for any of the hydrolysis components found to contain significant radioactivity in order to determine the complete labeling pattern for those compounds. Seven radioactively labeled compounds suspected of being precursors of viomycin were investigated with respect to their incorporation into viomycin.

Of major interest in this study was the biosynthetic origin of the viomycidine component in viomycin. *DL*-Arginine-amidine-^{14}C, *DL*-ornithine-1-^{14}C, and *DL*-arginine-1-^{14}C were chosen as possible viomycidine precursors. The resulting radioactively labeled viomycin samples were hydrolyzed; the individual hydrolysis components were separated.

Plots of the activities versus fraction number are shown in Figures 2, 3, and 4. The fractions containing the largest amounts of activity were identified by ninhydrin tests and by comparison of the data from previous experiments. The fractions combined, their identities, weights, and the total activities of the hydrolysis components are shown in Table 5.

Qualitatively, all three of these activity plots are similar in appearance, showing that each of the three precursors is incorporated into each of the hydrolysis components to approximately the same extent. All three precursors were incorporated primarily into isoviomycidine and viomycidine, as well as some of the more basic peptides. A total of 52.1 per cent of the total activity recovered from the Dowex 50 column was found in the isoviomycidine and viomycidine fractions originating from arginine-amidine-^{14}C precursor. When ornithine-1-^{14}C and arginine-1-^{14}C were used as precursors, 59.6 per cent and 77.2 per cent of the activities, respectively, were found in the isoviomycidine and viomycidine fractions.

In addition to these three compounds, *DL*-arginine-5-^{14}C was also used as a precursor in a previous experiment.[6] Again, significant activity appeared only in the isoviomycidine, viomycidine, and basic peptides fractions. Since these basic peptides are significantly labeled only in those experiments in which viomycidine is significantly labeled, these peptides apparently contain fragments derived from the viomycidine precursor.

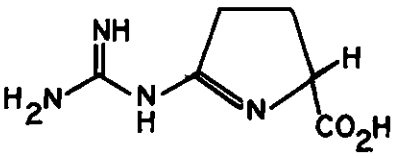
The fact that the three specifically labeled arginine precursors

and the ornithine precursor are each incorporated into viomycidine to a large extent is evidence that these compounds are direct precursors of viomycidine. This does not necessarily rule out that the X-amidinotransferase system proposed by Paśś and coworkers[38] is operating in viomycin biosynthesis, but it does seem to indicate that the system is of only minor importance, if it is operative at all.

It is interesting to note that both arginine and viomycidine are guanidino amino acids and that their molecular formulas differ by only four hydrogen atoms. The relationship between the structure of arginine (12) and that of viomycidine (4) is shown in Equation 2.*

Since the ornithine and arginine precursors were incorporated primarily into viomycidine, it was necessary to devise a suitable degradation scheme for viomycidine in order to determine whether the labeling pattern of the viomycidine was consistent with the labeling pattern of the precursor. Information obtained from such a study would indicate whether or not each precursor was incorporated specifically as the carbon skeleton of viomycidine.

The first structure proposed for viomycidine was 2-guanidino-Δ^1-pyrroline-5-carboxylic acid, 18.[56] The major evidence supporting this



18

*P. 16, this thesis.

structure was the fact that acetylation, followed by ozonolysis (hydrogen peroxide workup), and acid hydrolysis of the resulting material gave aspartic acid and guanidine.*

Viomycidine was treated with acetic anhydride and dry pyridine at steam bath temperatures for 8 to 20 hr. No attempt was made to isolate the acetylated viomycidine from the dark brown reaction mixture. Instead, the acetylated material was ozonized in 80 per cent formic acid at 0° for one hour. A 30 per cent solution of hydrogen peroxide was added to the ozonized solution, and the resulting mixture was allowed to stand at room temperature overnight. Acid hydrolysis of this material using 6 *N* hydrochloric acid and steam bath temperatures gave a mixture of *DL*-aspartic acid and guanidine in 52 per cent yield; traces of viomycidine and some other unidentified components were detected. The fact that *DL*- rather than *D*- or *L*-aspartic acid was obtained was attributed to racemization of the asymmetric center by pyridine during the acetylation.

Although no attempt was made to isolate an acetylated viomycidine from the pyridine-acetic anhydride reaction mixture, the compounds obtained from ozonolysis, followed by acid hydrolysis, suggest that the material present in the acetylation solution has the structure 19. Ozonolysis, followed by acid hydrolysis, would be expected to give guanidine from the guanidino group, aspartic acid from C-1, 2, 3, and

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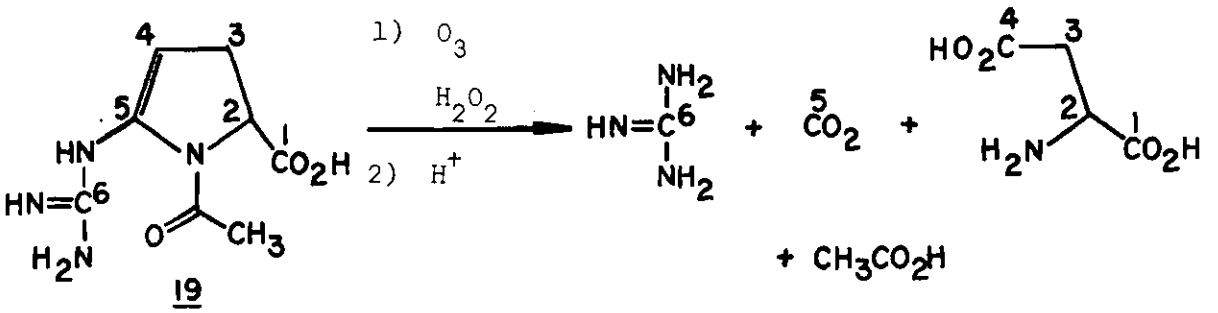
*Miller[57] prepared an acetyl derivative of viomycidine using acetic anhydride in ethanol-water solvent that was inert to ozonolysis. Acid hydrolysis of this derivative following ozonolysis gave only viomycidine and no significant degradation products.



19

4, and carbon dioxide from C-5.* The acetyl group would be converted to acetic acid in the acid hydrolysis.

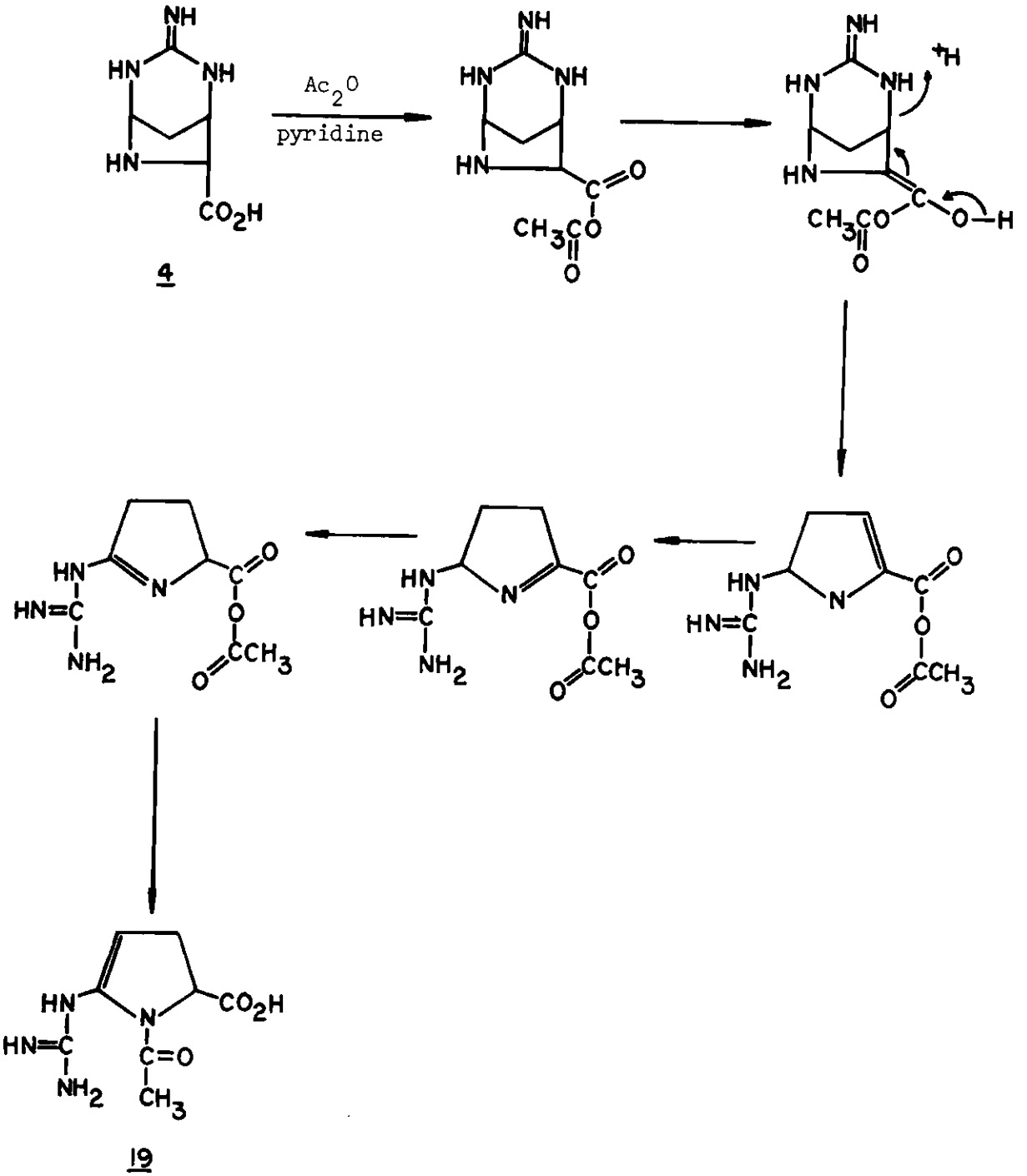
A possible mechanism for the formation of 19 in hot pyridine-acetic anhydride solution is shown in Scheme 1.[6]

The pyridine-acetic anhydride reaction sequence allows viomycidine to be degraded into individual compounds which, after separation of the hydrolysis components, contain only C-6, C-5, or C-1, 2, 3, and 4. The guanidine (C-6) and carbon dioxide (C-5) produced can be counted directly. The aspartic acid produced from C-1, 2, 3, and 4 can be oxidized with ninhydrin to separate C-1 from C-2, 3, and 4. Thus, the activities at C-1, C-2, 3, 4, C-5, and C-6 can be determined individually.

The degradations of the viomycidine samples from viomycin derived from arginine-amidine-^{14}C, ornithine-1-^{14}C, arginine-1-^{14}C, and arginine-5-^{14}C were carried out using the method developed by Nassar.[16] After the acetylation and ozonolysis (hydrogen peroxide workup) reactions, the solution was hydrolyzed using 6 *N* hydrochloric acid. The dark brown hydrolysate was decolorized with Darco G-60 and, after removal of the

*See footnote (p. 54, this thesis) for an explanation of the numbering system used.

Scheme 1



carbon, was evaporated to a syrupy, crystalline mass. This material was chromatographed using Whatman Number 17 preparative chromatographic paper and a 95 per cent ethanol:3 *N* ammonium hydroxide (5:1) solvent system. Only the centers of the aspartic acid and guanidine bands were cut from the paper strips in order to obtain the purest possible materials. This method separated the guanidine and aspartic acid components from each other and from most of the trace components very efficiently. Each sample was concentrated and diluted to exactly 3.00 ml with water for counting and further degradation studies.

Several problems are inherent in this degradation and separation scheme. None of the degradative reactions is quantitative in yield. A considerable amount of unchanged viomycidine remains in the degradation sample, and at least two other unidentified reaction products revealed by paper chromatography were present. Thus, the activities usually recovered in the aspartic acid, guanidine, and carbon dioxide (from C-5) are only a part of the total starting activity of each of the viomycidine samples. In addition, some overlapping of bands does occur on the paper chromatograms, and even selection of just the band centers does not avoid this problem completely. Of more importance is the fact that hot water elution of the guanidine and aspartic acid from the paper strips also yields relatively sizable quantities of soluble polysaccharides. Since the amounts of guanidine and aspartic acid separated by this method were usually small (5 to 20 mg), these water soluble saccharides presented a significant purity problem. No successful method of removing these impurities was found, and the impure guanidine and aspartic acid isolated were used without further treatment.

For these reasons, it was not possible to obtain accurate values for the specific activities of the guanidine and aspartic acid samples. Thus, no accurate comparison between the specific activities of the viomycidine samples and the samples of the degradation components could be made. It is interesting to note, however, that the activities isolated from each degradation were generally a constant fraction of the total starting activity. This indicates that the entire degradation and separation scheme resulted in approximately the same yield for each viomycidine sample.

These problems were at least partially overcome in the two experiments involving the ornithine-1-^{14}C and arginine-1-^{14}C precursors. A 250 µl aliquot of a 3.00 ml solution of the aspartic acid derived from the viomycidine degradation was counted in the usual way. A second 250 µl aliquot of the aspartic acid solution was subjected to ninhydrin oxidation. The activity contained in the first 250 µl aliquot was also recovered in the ninhydrin oxidation sample, either in the carbon dioxide trapped from the reaction, in the residual reaction mixture, or as the sum of the two. Thus, although accurate comparisons between the activities of the starting viomycidine samples and the guanidine and aspartic acid degradation products could not be made, the labeling pattern of the starting viomycidine could be inferred from the relative activities obtained for each degradation product. Comparison of the relative activities of each degradation component was also used to assign the labeling pattern to the viomycidine samples derived from arginine-amidine-^{14}C and arginine-5-^{14}C.

The viomycidine derived from the arginine-amidine-^{14}C precursor was degraded as previously described. No attempt was made to collect the carbon dioxide produced that was derived from C-5. The guanidine (C-6) isolated was found to contain 1820 dpm per 250 µl aliquot of a 3.00 ml solution of the crude guanidine isolate. The aspartic acid (C-1,2,3,4) isolated was found to contain 140 dpm per 250 µl aliquot of a similar solution of the aspartic acid isolate. Thus, approximately 92 per cent of the total activity isolated from the degradation scheme was found in the guanidine, while approximately 8 per cent of the activity was found in the aspartic acid. The aspartic acid sample was not degraded further.

Low levels of ^{14}C activity were found in almost all of the components isolated from this degradation scheme. As described previously, some overlapping of bands occurred in the paper chromatographic separation and thus, isolation of pure guanidine or pure aspartic acid was impossible. It is believed, therefore, that small activities found in samples such as the 8 per cent found in this aspartic acid sample result from ^{14}C-labeled impurities and not necessarily from labeling in the aspartic acid itself.

These results indicate that the guanidino group of the arginine precursor is incorporated into viomycidine without significant change. Since only a very small amount of the total activity isolated was found in the aspartic acid, the guanidine carbon is not appreciably scrambled with the other carbon atoms in viomycidine.

The viomycidine derived from the ornithine-1-^{14}C precursor was degraded and again, no attempt was made to collect the carbon dioxide from the C-5 carbon. The guanidine isolated was found to contain 113 dpm per 250 µl aliquot, or 3.1 per cent of the total activity. The aspartic acid was found to contain 3515 dpm per 250 µl aliquot, or 97.0 per cent of the activity. Ninhydrin oxidation of this aspartic acid sample was carried out in duplicate. An average of 1794 dpm per 250 µl aliquot of the aspartic acid was found at C-1 (48.6 per cent of the activity), while the residual ninhydrin oxidation reaction solution was found to contain 1788 dpm per 250 µl aliquot of the aspartic acid (48.4 per cent of the activity).

These results were rather surprising, since it was anticipated that ornithine-1-^{14}C would be incorporated specifically into viomycidine, such that only the carboxyl carbon of viomycidine (and thus the C-1 carbon of aspartic acid) would be significantly labeled. A small sample of the original ornithine-1-^{14}C precursor was subjected to ninhydrin oxidation in order to determine whether or not the ornithine was in fact specifically labeled at C-1.

Duplicate samples of the *DL*-ornithine-1-^{14}C used as the precursor for viomycin biosynthesis were degraded in the same manner as the aspartic acid. Counting of both the carbon dioxide produced and the residual reaction solution revealed that an average of 98.2 per cent of the ^{14}C activity was located at C-1 in the ornithine sample.

Due to the experimental difficulties previously described, it is impossible to interpret the data from this experiment conclusively. It

is possible that the chromatographic separation of the aspartic acid produced in the viomycidine degradation was not complete, and that some ^{14}C-labeled material that is not degraded by the ninhydrin oxidation reaction was present in the aspartic acid sample. Thus, the activity derived from the C-1 carbon of aspartic acid would be found in the carbon dioxide trapped, while the nondegradable material would remain in the reaction flask. It is probably coincidental that the numerical values for the activities were very nearly the same.

A second and more probable explanation is that the label of the ornithine-1-^{14}C is simply scrambled by some unknown biochemical pathway to give labeling at C-2, 3, and/or 4 of the aspartic acid resulting from degradation of viomycidine. Such a possibility is supported by the fact that all four of the degradation experiments were carried out in identical fashion and that both the aspartic acid derived from the arginine-1-^{14}C and that derived from the ornithine-1-^{14}C were degraded simultaneously and in exactly the same manner. It seems unlikely that an experimental inaccuracy of 50 per cent would occur under these conditions in only one of the cases. In addition, the activities for both aspartic acid samples were in good agreement with the starting activities of their respective viomycidine samples.

Since the results from this experiment are not conclusive, it is highly recommended that this experiment be repeated in a subsequent study on viomycin biosynthesis. Incorporation of other specifically labeled ornithine precursors should also be tried in order to clarify the biochemical fate of ornithine in viomycin biosynthesis.

The viomycidine derived from the arginine-1-^{14}C precursor was degraded; no attempt was made to collect the carbon dioxide from the C-5 carbon. The guanidine isolated was found to contain only 5.2 per cent of the total activity. The aspartic acid isolated was found to contain 94.8 per cent of the activity, of which 91.4 per cent of the total activity was at C-1 and 3.4 per cent was at C-2, 3, and 4.

These results suggest that the carbon skeleton of arginine is incorporated into viomycidine in viomycin biosynthesis. At least the carboxyl carbon of the arginine is incorporated as the carboxyl carbon of viomycidine with little or no scrambling.

It is interesting to note that if the X-amidinotransferase system proposed by Paśś and coworkers were operative, the arginine-1-^{14}C would be converted into an amidine group and ornithine-1-^{14}C. The amidine group may well be incorporated into viomycidine directly, but based on the results of the previous ornithine-1-^{14}C experiment, the labeling pattern of the ornithine would be expected to be scrambled before incorporation into viomycidine. Such is not the case, since arginine-1-^{14}C undergoes no scrambling upon incorporation into viomycidine. Thus, it is probable that the X-amidinotransferase system is inoperative in viomycin biosynthesis, and that arginine is incorporated directly into viomycidine.

It was anticipated that the majority of the ^{14}C activity in the viomycidine derived from arginine-5-^{14}C would be in the C-5 carbon of the viomycidine skeleton. This carbon is lost as carbon dioxide during the ozonolysis, workup, and acid hydrolysis steps of the degradation

scheme. Attempts were made to collect this carbon dioxide as the carbonate salt of Hyamine hydroxide and to count it directly. Although a total of 2.04×10^5 dpm of this activity was collected, this was only a small portion of the total starting activity.

Several experimental problems made the task of collecting the carbon dioxide quantitatively very difficult. The ozonolysis reaction apparatus was set up in such a way that the effluent gases from the ozonolysis mixture were bubbled through a 0°, liquid styrene trap, which removed all traces of residual ozone, and then into a trap containing Hyamine hydroxide. The gas flow that was necessary for complete ozonolysis of the acetylated viomycidine was much too rapid for the Hyamine hydroxide to effectively trap all of the carbon dioxide produced. It is possible that much of the ^{14}C-labeled carbon dioxide was lost in this step.

After ozonolysis, 5.0 ml of a 30 per cent solution of hydrogen peroxide was added to the ozonolysis solution in this closed reaction system through a rubber serum cap. The system was allowed to stand at room temperature overnight. After this time, 5.0 ml of additional hydrogen peroxide was added, and a very slow purge of oxygen was allowed to flow through the reaction system in order to collect any carbon dioxide produced in the workup. The Hyamine hydroxide trap, which was used for both steps, was diluted to 50.0 ml with methanol, and a 250 µl aliquot was counted as usual. A total of 8.7×10^4 dpm was collected from these reactions.

The reaction solution was removed from the ozonolysis system and

was evaporated to a syrup. No attempt was made to collect any carbon dioxide released during this evaporation due to the difficulties involved. The syrup was hydrolyzed using 6 *N* hydrochloric acid as usual. A slow nitrogen purge was bubbled through the hydrolysate and into a second Hyamine hydroxide trap. The contents of the trap were diluted and counted as before. A total of 1.2×10^5 dpm was collected from the acid hydrolysis reaction.

The aspartic acid isolated from the acid hydrolysate had an activity of 170 dpm per 250 µl aliquot and the guanidine isolated had an activity of 13 dpm per 250 µl aliquot. Although these activities are not directly comparable to the activity of the carbon dioxide collected, it is readily apparent that far less than 1 per cent of the total activity is located at C-1, 2, 3, 4, and 6.

From these data, it is apparent that the C-5 carbon in the arginine skeleton is incorporated into the C-5 carbon of the viomycidine skeleton with almost no scrambling.

Although the results of the ornithine-1-^{14}C experiment are not conclusive, the results of the three arginine experiments strongly suggest that arginine, at least, is incorporated directly into the viomycidine unit in the viomycin molecule. No alteration of the carbon skeleton of the arginine is evident in any case. It is recommended that future studies on these biosynthetic pathways should include experiments using several labeled ornithine and citrulline precursors, as well as other metabolic products of arginine.

Since the reaction pathways for viomycin biosynthesis were

unknown, it was important to devise experiments that would indicate the overall route of the biosynthesis of viomycin. At least two reaction pathways were considered: 1) that a polypeptide chain of normal amino acids was constructed and then modified biosynthetically to give the unusual amino acid residues, or 2) that the unusual amino acids were biosynthesized first and then incorporated into the viomycin polypeptide chain. In order to test these possibilities, it was necessary to attempt to incorporate ^{14}C-labeled samples of the unusual amino acids α,β-diaminopropionic acid, β-lysine, and viomycidine directly into viomycin. Thus, if the first pathway were operative, the ^{14}C label from each precursor would either not be incorporated at all, or would be distributed among several of the amino acid residues of viomycin. If the second pathway were operative, each of the precursors would be expected to be incorporated directly into the respective residue in viomycin with no scrambling of the ^{14}C label or distribution into the other amino acids.

Samples of ^{14}C-labeled α,β-diaminopropionic acid, β-lysine, and viomycidine are unavailable commercially. Since it was desirable to use these compounds as potential precursors for viomycin biosynthesis, the glucose-U-^{14}C experiment (Run No. 1) carried out by Floyd[6] was repeated using a much larger quantity of more highly labeled glucose. In this experiment (Run No. 18), viomycin was produced in the usual manner. The viomycin was hydrolyzed without the addition of unlabeled viomycin, and the hydrolysate was chromatographed in the same manner as that of the previous experiments. The activity curve for this

experiment is shown in Figure 5. The four peaks centered at fractions 162, 304, 321, and 357 were isolated and identified as serine, diaminopropionic acid, β-lysine, and viomycidine, respectively. A number of other unidentified peaks of activity were also found. It is noteworthy that even in this experiment, in which the levels of activity were greater than in any other experiment, not a trace of urea was found in the hydrolysate of the viomycin. The small peak centered at fraction 98 gave a negative *p*-dimethylaminobenzaldehyde test, showing that no urea was present. The diaminopropionic acid, β-lysine, and viomycidine fractions were separated, lyophilized, and used for incorporation without further purification.

The ^{14}C-labeled diaminopropionic acid from Run No. 18 was incorporated into viomycin (Run No. 19). The resulting viomycin was hydrolyzed, and the hydrolysate was chromatographed as usual. A plot of the activities versus fraction number is shown in Figure 6. The peak centered at fraction 150 was determined to be serine, and the peak at 284 was α,β-diaminopropionic acid.

Floyd[6] has previously determined that diaminopropionic acid from viomycin derived from glucose-U-^{14}C was uniformly labeled. The diaminopropionic-^{14}C acid from Run No. 19 was also degraded to determine the labeling pattern. Ninhydrin oxidation revealed that 27.6 per cent of the total activity was located at C-1; periodate oxidation revealed that 33.2 per cent of the activity was located at C-3; by difference, 39.2 per cent of the activity was at C-2. These data are shown in Table 6 and indicate within experimental error that the diaminopropionic acid

from viomycin derived from the diaminopropionic-U-^{14}C acid precursor is also uniformly labeled.

It is noteworthy that while Floyd's studies[6] showed that specifically labeled serine was converted to diaminopropionic acid to a significant extent, this study showed that the reverse conversion is of only minor importance, since very little ^{14}C-labeled serine was produced. Neither is the diaminopropionic-U-^{14}C acid apparently converted biosynthetically into any of the other hydrolysis products, for no other peaks of activity are found in the activity curve (Figure 6).

The β-lysine-^{14}C from Run No. 18 was also incorporated into viomycin (Run No. 20). A plot of the activities versus fraction numbers from the hydrolysis and chromatography of this viomycin sample is shown in Figure 7. The peak centered at fraction 400 was positively identified as β-lysine by tlc, paper chromatography, and paper electrophoresis (0.2 *M* formic acid buffer). From the activity curve (Figure 7), it is readily apparent that β-lysine precursor is incorporated only into β-lysine in viomycin biosynthesis. An almost total absence of other activity peaks rules out any conversion of β-lysine into other components of viomycin. In this way, β-lysine was found to be quite different biosynthetically from lysine, which was significantly converted into serine and diaminopropionic acid in previous studies using lysine-2-^{14}C[6] and lysine-1-^{14}C.[14]

Although viomycidine does not occur in viomycin, an attempt was made to incorporate the viomycidine-^{14}C from Run No. 18 into viomycin. An activity curve from the hydrolysis and chromatography of this

viomycin sample (Run No. 21) is shown in Figure 8. Apparently, the viomycin-producing organism cannot use viomycidine very efficiently as a precursor for the synthesis of viomycin since only minor activity was found in any fraction. Thus, this study is further evidence that viomycidine does not exist in viomycin *per se*, but is produced during acid hydrolysis of viomycin.

These studies show very conclusively that viomycin is biosynthesized by prior formation of the unusual amino acids, followed by direct incorporation of these amino acids into the viomycin polypeptide chain. The fact that each of these amino acids is incorporated only into the respective residue in viomycin suggests that the biosynthetic reactions that produce these unusual amino acids are essentially irreversible. In addition, each of the unusual amino acids was incorporated into viomycin at least to the same extent that other more common precursors were incorporated. The β-lysine precursor was incorporated into viomycin to a greater extent (8.15%) than any other ^{14}C-labeled precursor used.

In addition to the ^{14}C-labeling experiments, three other experiments using ^{15}N- or deuterium-labeled precursors were carried out. A sample of 3,3-dideuterioserine (90% 2 ^{2}H at C-3) was incorporated into viomycin (Run No. 24), and the viomycin produced was hydrolyzed. No unlabeled viomycin was added to the labeled sample prior to hydrolysis. The hydrolysate was separated into the individual components as usual. Low resolution mass spectra were obtained for the serine and diaminopropionic acid samples from this experiment. The serine samples had an

indicated atom excess of 1.32 per cent at M + 1 (^{2}H) and 0.65 per cent at M + 2 (2^2H) for the molecular ion (m/e 60,61,62). The diaminopropionic acid sample was found to have an indicated atom excess of 6.15 per cent at M + 1 (^{2}H) and 1.78 per cent at M + 2 (2^2H) for the molecular ion (m/e 59,60,61).

These results indicate that both the serine and diaminopropionic acid samples have two deuterium atoms per molecule, as did the original precursor. This would indicate that serine is aminated directly to produce diaminopropionic acid, rather than oxidized to the semialdehyde and transaminated as proposed by Reinbothe.[29]*

Lysine-^{15}N (Run No. 22) was also studied in order to determine whether or not the nitrogens are incorporated directly into the other amino acids without the intervention of free ammonia. The lysine-^{15}N (95 atom per cent ^{15}N in both positions) was incorporated into viomycin. After hydrolysis and separation of the hydrolysate, the serine, diaminopropionic acid, and β-lysine produced were analyzed by low resolution mass spectrometry. The serine derived from lysine-^{15}N showed a 1.66 per cent atom excess at M + 1 (m/e 61), which indicates that one of the nitrogens of lysine is incorporated directly into serine. Based on previous ^{14}C-labeling experiments, it is quite evident that the carbon skeleton of lysine is also incorporated directly into serine. Thus, it would appear that C-1, 2, and possibly C-3 of lysine are converted into serine, without loss of the C-2 amino group.

The diaminopropionic acid derived from the lysine-^{15}N is probably

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*See Equation 1, p. 13 this thesis.

produced in a similar manner, since 0.97 per cent atom excess was found at C-2 of diaminopropionic acid, and 0.85 per cent was found at C-3. These results indicate that both nitrogens of diaminopropionic acid are labeled to approximately the same extent.

Although the data obtained from these low resolution mass spectra may not be precise, it is believed that at least a good indication of the labeling pattern can be obtained. High resolution mass spectra are being obtained; these will give a much more accurate indication of the labeling pattern of these samples.

Although no mass spectral data on β-lysine has been reported in the literature, the fragmentation pattern was found to be analogous to that of lysine.[58,59] Two peaks in the β-lysine spectrum were studied; the peak at m/e 56 corresponds to $H_2\overset{+}{N}{=}CHCH{=}CH_2$, which represents only the nitrogen contained in the ε-amino group of β-lysine. The peak at m/e 101 represents the diamine ion $H_2N\text{-}CH_2CH_2CH_2\overset{NH_2}{\overset{|}{C}}HCH_2^+$, which includes both the β- and ε-amino groups. Thus, the atom excess at the β-position was indicated by the difference in the atom excess at m/e 103 and m/e 57.

The β-lysine derived from lysine-^{15}N was found to be labeled in both the β- and the ε-positions. It appears, therefore, that the amino groups of lysine that are incorporated into β-lysine are not converted to ammonia. In conjunction with the ^{14}C-labeling results, it is clear that lysine is incorporated directly into β-lysine without prior metabolism. These results are in agreement with those reported by Barker and coworkers[34] and by Stadtman and coworkers.[35]

Serine-^{15}N was also used as a precursor for viomycin biosynthesis (Run No. 23). The viomycin produced was hydrolyzed, and the hydrolysate was separated as usual. Low resolution mass spectra were obtained for the serine, diaminopropionic acid, and β-lysine samples from this experiment. As anticipated, both serine and diaminopropionic acid were labeled; however, the diaminopropionic acid appeared to be labeled approximately equally at both the α- and β-positions. This would suggest that diaminopropionic acid is produced from serine by direct amination involving a second molecule of serine as the nitrogen donor. Intervention of free ammonia would be expected to result in little or no labeling at the β-position and thus, direct amination involving free ammonia could not account for the observed results.

The β-lysine derived from this experiment was found to be labeled almost exclusively at the β-position. These data are in agreement with the ^{14}C-labeling experiments and indicate that serine is a precursor of β-lysine.

CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS

Viomycin can be catalytically hydrogenated to give a homogeneous perhydro product that differs from the parent viomycin in amino acid composition. The perhydroviomycin has no uv chromophore, but is biologically active. The data from these experiments were used to invalidate the structures previously proposed for viomycin. It is recommended that further structural studies of viomycin be pursued, especially in the direction of a single crystal X-ray analysis of a suitable derivative of viomycin. It is also recommended that further study of the perhydroviomycin be pursued in light of the biological activity that this material demonstrated.

Seven radioactively labeled compounds were studied as possible precursors for viomycin biosynthesis. Arginine-amidine-^{14}C, ornithine-1-^{14}C, arginine-1-^{14}C, and arginine-5-^{14}C were found to be incorporated mostly into the viomycidine unit of viomycin. Degradation of each viomycidine sample derived from each of these precursors revealed that each viomycidine sample retained the same labeling pattern as the respective arginine precursor, but the label from the ornithine-1-^{14}C precursor appeared to be scrambled.

α,β-Diaminopropionic-U-^{14}C and β-lysine-^{14}C (labeling pattern undetermined) were also studied as potential precursors for viomycin biosynthesis, and both were found to be incorporated only into their

respective residues in viomycin. Thus, no conversion of either compound occurs in *S. griseus* that would result in labeling in any of the other amino acids of viomycin. Viomycidine-^{14}C was not incorporated into any component of viomycin to a significant extent.

Lysine-^{15}N, serine-^{15}N, and 3,3-dideuterioserine were all found to be incorporated into viomycin. From the lysine-^{15}N, both nitrogen atoms of β-lysine and diaminopropionic acid and the nitrogen atom of serine were found to be significantly labeled. Thus, lysine would seem to be converted directly into β-lysine without intermediate conversion of the amino nitrogen atoms into free ammonia. The dual labeling of the diaminopropionic acid would appear to indicate that this compound is produced biosynthetically from a serine residue by direct amination involving a second serine molecule. The ^{15}N-labeling in serine is in agreement with at least one known pathway of lysine to serine conversion.

The serine and diaminopropionic acid derived from the 3,3-dideuterioserine were both found to contain two deuterium atoms per molecule. This indicates that in the serine to diaminopropionic acid conversion, the C-3 carbon of serine is not converted to an aldehyde and then aminated, but is apparently aminated by direct displacement, such that neither of the deuterium atoms is displaced.

It is strongly recommended that the ornithine-1-^{14}C labeling experiment be repeated. The results from this experiment were somewhat inconclusive, and clarification of the biosynthetic incorporation of ornithine into viomycin in *S. griseus* is necessary. Other specifically

labeled ornithine precursors, as well as some specifically labeled citrulline precursors should be studied in order to clarify the pathway of incorporation into viomycin of these compounds. Also of interest would be an attempt to incorporate ^{14}C-labeled capreomycidine into viomycin in order to determine whether this material is incorporated into the pre-viomycidine unit in viomycin.

Research topics of further interest might include a ^{14}C-labeling study of perhydroviomycin. Several of the biosynthetic experiments already completed should be repeated, and the viomycin produced should be catalytically hydrogenated. Hydrolysis and separation of the hydrolysis components should reveal information about the nature of the pre-alanine unit, the pre-viomycidine unit, and the source of the carbon dioxide released during acid hydrolysis.

APPENDIX

SOURCES OF PRECURSORS AND PRODUCTION DATA FOR VIOMYCIN BIOSYNTHESIS

Sources of Precursors for Viomycin Biosynthesis

Run No.	Precursor	Source
15	*DL*-Arginine-amidine-^{14}C	New England Nuclear Corp., Boston, Mass.
16	*DL*-Ornithine-1-^{14}C	New England Nuclear Corp., Boston, Mass.
17	*DL*-Arginine-1-^{14}C	Schwarz Bioresearch Inc., Orangeburg, N. Y.
18	*D*-Glucose-U-^{14}C	New England Nuclear Corp., Boston, Mass.
19	*L*-α,β-Diaminopropionic-U-^{14}C Acid	Derived from Run No. 18
20	*L*-β-Lysine-^{14}C (labeling pattern undetermined)	Derived from Run No. 18
21	*L*-Viomycidine-^{14}C (labeling pattern undetermined)	Derived from Run No. 18
22	*L*-Lysine-^{15}N	Schwarz Bioresearch Inc., Orangeburg, N. Y.
23	*L*-Serine-^{15}N	Schwarz Bioresearch Inc., Orangeburg, N. Y.
24	3,3-Dideuterioserine	Gift from Dr. C. C. Sweeley, Michigan State University

Production Data for Viomycin Biosynthesis

	1.	2.	3.	4.[a]	5.	6.[b]
Run No.	Precursor	Precursor Added	Precursor Specific Activity (dpm/ μmole)	Viomycin Produced (mg)	Carrier Added (mg)	Viomycin Total (mg)
15	*DL*-Arginine-amidine-^{14}C	4.93×10^{8} dpm	6.17×10^{6}	323	0.0	323
16	*DL*-Ornithine-1-^{14}C	6.86×10^{8} dpm	2.79×10^{6}	247	0.0	247
17	*DL*-Arginine-1-^{14}C	7.06×10^{8} dpm	2.53×10^{7}	213	0.0	213
18	*D*-Glucose-U-^{14}C	2.16×10^{10} dpm	9.73×10^{7}*	959	0.0	959
19	*L*-α,β-Diamino-propionic-U-^{14}C Acid	2.09×10^{7} dpm	1.54×10^{4}	165	0.0	165
20	*L*-β-Lysine-^{14}C (labeling pattern undetermined)	1.43×10^{7} dpm	1.19×10^{4}	225	0.0	225
21	*L*-Viomycidine-^{14}C (labeling pattern undetermined)	6.92×10^{6} dpm	1.24×10^{4}	307	0.0	307
22	*L*-Lysine-$^{15}N\cdot HCl$	0.296 mmole	-	108	0.0	108
23	*L*-Serine-^{15}N	0.438 mmole	-	213	0.0	213
24	3,3-Dideuterioserine	0.762 mmole	-	187	0.0	187

*Includes total glucose in growth medium.

[a]Column 4 based on preliminary assay prior to addition of carrier.

[b]Column 6 based on effluent from preparative IRC-50 column.

Production Data for Viomycin Biosynthesis
(Continued)

Run Number	7.[c] Pure Viomycin Recovered (mg)	8. Per Cent Viomycin Recovered (7./6.)	9. Viomycin Recovered Actual Weight (mg)	10. Viomycin Per Cent Purity (7./9.)	11. Viomycin Total dpm Isolated
15	181	56.1	204	88.8	1.49×10^{7}
16	172	69.5	220	78.0	1.10×10^{7}
17	152	71.5	171	89.0	1.18×10^{7}
18	870	91.7	1065	81.7	1.55×10^{8}
19	147	89.1	188	78.2	6.97×10^{5}
20	205	91.2	260	78.9	1.07×10^{6}
21	256	83.5	321	79.8	7.58×10^{4}
22	71	65.7	88	80.8	-
23	142	66.7	191	74.3	-
14	114	61.0	173	66.0	-

[c]Column 7 based on spectrophotometric assay of final product.

Production Data for Viomycin Biosynthesis
(Continued)

	12.	13.	14.
Run Number	Viomycin Total dpm Produced (11./8.)	Per Cent Incor-poration (12./2.)	Viomycin Specific Activity (dpm/ μmole)
15	2.65×10^{7}	5.40	6.21×10^{4}
16	1.59×10^{7}	2.32	4.85×10^{4}
17	1.65×10^{7}	2.34	5.85×10^{4}
18	1.69×10^{8}	0.78	1.35×10^{5}
19	7.82×10^{5}	3.74	3.57×10^{3}
20	1.17×10^{6}	8.15	3.92×10^{3}
21	9.08×10^{4}	1.31	2.23×10^{2}
22	-	3.54[d]	-
23	-	2.26[d]	-
24	-	1.38[d]	-

[d] Values based on previous experiments using ^{14}C-labeled precursors.

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